

# University challenge for Britain

**Sustaining a strong and competitive ability in research, as in sport, requires sustained commitment to investment in infrastructure. The British government risks compromising the prospects for long-term scientific achievement.**

BRITAIN'S dismal performance in the Olympic Games — its performance in athletics, for example, was the worst since the 1952 games in Helsinki — has come as a brutal reminder of the harshness of international competition in the modern world. Several lessons can be drawn. One is that era of the gifted amateur, celebrated nostalgically in the film 'Chariots of Fire', has truly ended. A second is that modern athletes can no longer hope to succeed without the backing of a solid infrastructure that allows them to develop their talents to the full. Finally, most of the countries that have returned from Atlanta with the largest collection of medals are the ones that have accepted the need for substantial and sustained public investment in such infrastructure, and the limits to private finance in achieving this goal.

Each of these lessons could be applied instructively to the plight of British universities, and in particular to that of research within these institutions. There was a time when Britain rode high in the league of Nobel prizewinners, on the basis partly of the undoubted strengths of an élitist educational system. But those days are past. In the future, economic success — and the scientific success on which this will increasingly depend — will require substantial investment not only in individuals but also in the physical resources which high-quality teaching and research require. For this, adequate public funding, seen as a capital investment rather than a recurrent expense, is essential.

At present, the British government appears reluctant to accept this reality. To some extent, its reaction is understandable; faced with economic and political pressures to keep a tight cap on public spending, and aware that there are many fewer votes to be lost by squeezing universities than by raising taxes, it has inevitably been tempted to pursue its fiscal objectives partly by reducing its financial commitment to higher education. This has led to some dramatic moves. In last November's budget for the fiscal year 1996–97, for example, which began in April, the government announced a 31 per cent cut in capital funding for higher education — a sum of about £300 million — compared to the previous year. In doing so, it made clear that, where possible, investment capital should be raised through private-sector schemes in line with the government-backed Private Finance Initiative (PFI).

But, as the government appears to be recognizing belatedly in its attitude to the funding of sport, this approach is misguided. There are undoubtedly some forms of investment by universities and other higher education institutions — for example, in student accommodation or in teaching blocks — where the possibility of using these to generate additional income makes private financing a viable option. But much of this was happening anyway. And as even the report of a working group set up jointly by the Department for Education and Employment and the higher education sector (DfEE/HE), published last week, makes clear, there are other areas, particularly in the provision of specialized research and teaching equipment, where a PFI approach is "difficult to apply and time-consuming to pursue".

The needs for such investment are clear. They were, for example, dramatically highlighted in a report commissioned earlier this year by the Committee of Vice-Chancellors and Principals (CVCP), the body that represents higher education institutions in their negotiations with government, from the Policy Research in

Engineering, Science and Technology institute and the Centre for Applied Social Research at the University of Manchester. This found that four-fifths of university departments covered in a survey reported important research areas where a lack of funding for equipment was holding back progress. Nearly a fifth of research equipment was found to be of poor technical capability, and the report pointed out that some large companies are already seeking collaborators in academic institutions abroad because of the decay in the UK academic infrastructure.

There are, of course, dangers in overstating the plight of British universities. Although the CVCP, for example, has been able to point to a long list of university construction projects delayed partly because of a lack of adequate public finance, building work at other institutions is continuing apace. Furthermore, university science faculties have, through force of necessity, become increasingly skilled at using their entrepreneurial talents to raise funds for equipment and new buildings in a way that the government enthusiastically endorses. Indeed, the Manchester report found that more than half of university science departments in Britain felt that their equipment is as good as, or better than, the international average.

But there are many worrying aspects to current trends. Much of the new money that has gone into major construction projects in the biomedical sciences over the past decade, for example, has come from the Wellcome Trust, the richest research foundation in the world following the recent sale of its shares in the Wellcome pharmaceutical company (merged last year with Glaxo). Yet, as the trust itself properly insists, it is keen that this money should be used to complement public funding, not to replace it. This strategy is all the more important at a time when public investment in science is increasingly geared towards strategic economic goals, with the danger of broader considerations being forgotten.

Furthermore, there are areas in which relying on the type of PFI schemes suggested by the government can have its own drawbacks. Take information technology (IT), for example. This is one area that the DfEE/HE working party endorsed as having significant potential for growth, pointing out that in some universities, spending on IT can consume 70 per cent or more of equipment grants. Government officials emphasize the potential value of entering long-term commitments with major suppliers of such equipment, based on borrowed capital. But many universities are wary of this approach, pointing to the dangers of locking into a single supplier, as many schools have found, in a field where the leading-edge technology is changing rapidly.

As in sport, private sponsorship has an important role to play; but it is not the answer to sustained, long-term achievement. The government needs to recognize this fact, and to draw up a new strategy for supporting the research base in universities based on the concept of public, not private, investment. This will undoubtedly require some creative thinking, much of it hopefully focused within the working party now studying the future shape of UK higher education, chaired by Sir Ron Dearing. It will also, as in sport, pose some difficult dilemmas, such as the need to focus resources selectively on high performers without falling prey to élitism. But without a fundamental change in thinking, British science, like British sport, risks sinking gently into oblivion. □



# Lack of funds for technology 'could undermine' Mars mission science

**Washington.** An ambitious US plan to launch spacecraft to Mars every two years could be hampered by a lack of funding for new technology, according to both a National Research Council report and scientists and contractors familiar with the proposed missions.

Unless NASA invests more money in miniaturized scientific instruments, large roving vehicles and other key technologies, the proposed Mars missions may have their scientific objectives "seriously undermined", according to a report to be released this week by the Committee on Planetary and Lunar Exploration (COMPLEX).

The committee, chaired by Ronald Greeley of Arizona State University, is particularly concerned about funding for scientific 'microinstruments', which it says has not kept up with spacecraft design.

The panel also says that answering key questions about Mars — particularly conducting a comprehensive search for existing or fossil life — will require larger and more capable landers and roving vehicles than those NASA now has in mind. 'Minirovers' of the type that the Mars Pathfinder spacecraft will land on the planet's surface next July have a limited range, measured in tens of metres, and can last only a few weeks.

The goals and design of NASA's Mars Surveyor programme, which will spend about \$1 billion over the next decade to visit Mars at each 26-month opportunity, have wide support among space scientists. But many say they are concerned that the programme is underfunded.

Daniel Goldin, the administrator of NASA, only added to such concerns by challenging his engineers, at a recent symposium to mark the 20th anniversary of Viking's landing on Mars, to return a sample of the planet as early as 2003. A sample return was not part of the original Mars Surveyor plan. Most observers, inside and outside NASA, doubt whether it can be accommodated within the existing budget.

"Money is the key to this programme," says Donna Shirley, Mars exploration manager at the Jet Propulsion Laboratory (JPL). Shirley boasts in her public presentations that the \$150 million price of each Surveyor mission is about the same as that of a Hollywood blockbuster film, and that the agency is exploring Mars for \$300,000 a day.

But, speaking at the Viking symposium, Noel Hinners warned that the agency's investment in technology was "totally inadequate" to support the ambitious missions

planned for 2001 and after, particularly if the sample return is thrown in. Hinners is a former head of NASA's science office and now vice-president of flight systems at Lockheed Martin Corporation, which is building the 1996 and 1998 Surveyor spacecraft for NASA.

One possible way of affording a sample return while remaining within the Surveyor budget would be to scale down earlier missions in the series. But a recent analysis at JPL, which Shirley stresses was only preliminary, suggests that even this would not make up the shortfall. Bringing in international partners would be another possible solution. NASA is already talking to the Russians about a joint Mars mission in 2001.

NASA's human exploration office might also be able to contribute funds. But many technical and financial hurdles would still remain. One critical issue, says Shirley, will be arranging for quarantine of any samples returned from Mars, to avoid any risk of "back contamination".

Both the US missions heading for Mars

this autumn will have substantial risks attached, largely as a result of their constrained budgets. The Mars Pathfinder, which is a technology test rather than a science mission, relies on an airbag system which has never previously been used to protect its lander on impact with the Martian surface. To save money, many of the on-board systems have no back-up.

The Mars Global Surveyor, which is due to be launched in December and duplicates much of the failed 1994 Mars Observer mission, will depend on a risky, months-long 'aerobraking' manoeuvre to decelerate in the Martian atmosphere. Russia's Mars 96 mission will also be launched in November, with an orbiter and two landers on board.

NASA's emphasis on economy bothers some engineers from the old school. Others are simply holding their breath. Louis Friedman, who follows Mars exploration as executive director of the Planetary Society, describes the forthcoming Mars 'Derby' as a "turning point" for the 'cheaper, better, faster' approach. **Tony Reichhardt**

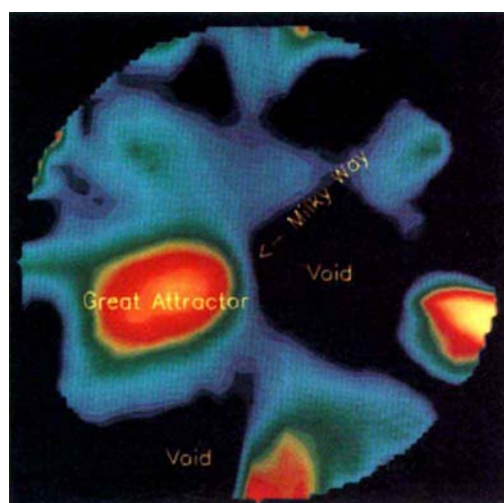
## Space voids really are devoid of matter

**London.** A team of astronomers from Australia, Chile, Europe and the United States has discovered that the large empty regions in space, known as 'holes' or 'voids' (see right), are literally devoid of matter.

Astronomers have presumed for some time the existence in the Universe of 'dark matter', which cannot be seen but whose presence can be traced by its gravitational effect on visible matter, such as stars and galaxies. The newly-discovered lack of dark matter within voids indicates that it is not smoothly distributed.

Astronomers from the European Southern Observatory (ESO) have now devised a technique that attempts to 'flush out' this 'invisible' matter, if there is any. Using optical and radio telescopes around the world, they measured the speeds of 2,000 well known galaxies. They then estimated the distribution of these galaxies on the basis of their observed velocities and positions.

Assuming interactions only through



gravity, the astronomers estimated where the dark matter would have to be to produce the distribution and velocities of the real galaxies. To their surprise, they found several voids that do not need any matter at all.

The picture above is a simulation of these empty voids in the local Universe. The analysis will be published in the 1 September issue of *Astrophysical Journal Letters*. □



# Future of energy labs under further scrutiny

**Washington.** The US Department of Energy has started yet another round of reviews of its sprawling network of research laboratories, deferring any action to streamline them until after November's presidential and congressional elections.

Announcing the reviews last week, Charles Curtis, the deputy energy secretary, ruled out the closure or consolidation of any of the larger laboratories. He added that privatization would only be considered for seven small ones, each with annual budgets of less than \$50 million.

Republicans in Congress say that a preliminary plan for the laboratories released by the Department of Energy falls considerably short of the strategic plan that the department promised to deliver this year. But the Congress will be in session for only one more month — September — this year, and will not revive

the issue of laboratory reform until 1997.

Curtis announced four new reviews of the laboratory network, which employs more than 50,000 people in centres ranging from the Los Alamos nuclear weapons laboratory in New Mexico to the newly opened Thomas Jefferson accelerator laboratory in Virginia. The network houses most large physics experiments in the United States, has unmatched expertise in nuclear weapons design, supercomputer use and materials science, and significant activity in most other scientific disciplines.

The first review is to be completed by the energy department's Laboratory Operations Board by 1 November. It will assess whether the department's programme managers are making the right choices between the laboratories and other options, such as universities, in deciding where to do research.

In a second review, next year, the board

will examine the possible privatization of the seven smallest single-purpose laboratories. Last week one such laboratory — the National Institute for Petroleum and Energy Research at Bartlesville, Oklahoma — was singled out for privatization in 1998.

The third review will deal with the strategic plans of the nine large, multi-purpose laboratories. A final review, to be conducted by the outside members of the operations board (which mixes senior civil servants with outside experts), will assess how peer review is applied to research programmes.

Curtis was asked why the Clinton administration was ending four years of alleged effort to reform the laboratories with the promise of yet more reviews. He said that the review process would survive any change of administration, as it drew on the most senior civil servants in the department and outside expertise from individuals with various political views. The board's vice-chairman is John McTague of Ford, a former adviser to President Ronald Reagan.

"We've made a concerted effort to make sure that this work will extend from one administration to the next," Curtis said. He added that the current review process and the preliminary plan were different from previous efforts, because they "follow the money" in the laboratory network. He said that "the most important review" was the first one, which would be ready in time to influence the first budget proposal of the next administration, due next February.

Early in the life of this administration, Hazel O'Leary, the energy secretary, asked Bob Galvin, an executive with Motorola, to head a task force to review the laboratories. In February 1995, Galvin issued a scathing report that called for radical changes in the governing structure of the laboratories; efficiency improvements to cut costs by one-third; and the ending of independent nuclear weapons design at the Lawrence Livermore laboratory in California (see *Nature* 373, 463; 1995).

Galvin's proposed changes attracted early interest from Republicans in Congress, who sought to begin reform at the top by abolishing the Department of Energy. Some congressmen called for a "closure commission" to shrink the laboratory network. But senior Republicans such as Senator Pete Domenici (New Mexico), chair of the Budget Committee and energy appropriations subcommittee in the Senate, succeeded in defending the department and its laboratories.

According to congressional staff, however, the issue of abolishing the department and reforming the laboratories will be revived next year if Republicans retain control of Congress. One staff member doubted that Domenici will fight as hard to defend the laboratories once he achieves his goal of re-election in November. **Colin Macilwain**

## Standards buildings fight for funds

**Washington.** Prospects are fading for badly needed renovations and new facilities at the US National Institute of Standards and Technology (NIST), formerly known as the National Bureau of Standards. The agency finds itself in a losing battle with Congress over the way it handled its construction funds last year.

A \$540-million renovation plan for NIST's two main sites, at Gaithersburg, Maryland, and Boulder, Colorado, is already being revised. The plan sank deeper into trouble last week, when Senate appropriations committee members joined their counterparts in the House of Representatives in rejecting an administration request for \$105 million towards the plan next year.

The agency started work on 29 July on a \$57-million chemistry laboratory at Gaithersburg (pictured). This project had become NIST's top priority because of deterioration in the existing laboratory, aggravated by asbestos problems. The work is likely to be completed on schedule in 1998.

But more ambitious plans for state-of-the-art metrology laboratories on both sites, along with the renovation of facilities built between 30 and 45 years ago, seem unlikely to proceed, after the Senate appropriations committee offered NIST \$15 million for construction next year "for maintenance and safety".

Senate appropriators also allocated \$60 million for the Advanced Technology

Programme (ATP), which is strongly favoured by President Bill Clinton. The House had offered \$110 million, against \$345 million requested by Clinton. But the Senate rejected a House provision that would have ejected medium-sized and large companies from the programme.

The standards-related work of NIST has strong bipartisan support, and continues to attract full funding from the Congress. But the removal of the construction funds, which would have financed an overall plan forged under the Republican administration of President George Bush, indicates that the agency is suffering in Congress for its association with the ATP.

Connie Morella (Republican, Maryland), chair of the technology subcommittee of the Science Committee in the House of Representatives, who has NIST's Gaithersburg site in her district, says that she will continue to fight for the new laboratories.

Morella says that support for NIST's core programme remains solid, and construction funds could be restored if appropriators and the agency can settle an arcane argument about how the agency classified some money that Congress sought to cut from its budget last year. "I'm hoping we can smooth things over," Morella said before last week's Senate action, which makes the hope look forlorn, this year at least. **C. M.**





## Peer review deemed essential for 'blue skies' award scheme

London. Britain's Royal Society has given lukewarm support to Realizing Our Potential Awards (ROPA), a two-year-old government scheme to reward 'blue skies' researchers who collaborate with industry.

The society's report, published this week, endorses the general goal of the scheme. But it says that so far it seems to be having little impact on the volume of links between industry and academic institutions.

Nevertheless the Royal Society says that the ROPA scheme is "an important experiment" in public funding of science and should continue, but without any additional funds. The report says that the proportion of research council funding for universities to ROPAs should not exceed the present nine per cent until the quality of projects can be established.

Much of the report is taken up with an assessment of the government's decision not to use peer review to assess ROPA-funded projects, but to rely on evaluation by panels of industrialists. Announcing the ROPA scheme in February 1994, William Waldegrave, then Cabinet minister for science, said research proposals would by-pass peer review on the grounds that the system "disadvantaged" the types of speculative or unorthodox proposals the government was keen to encourage.

But the Royal Society report concludes that this alternative system appears to have failed, and supports the "full use of scientific referees". The authors say members of the panels of industrialists "felt restricted by not being allowed to give the same detailed scientific scrutiny to ROPA applications as was given to other [research funding] applications". In addition, evidence seemed to show that only a small proportion of speculative fundamental research projects had been funded.

The report also calls for an independent evaluation of the quality of ROPA proposals — once the project results are published — to allay fears that funds have been awarded to "work of second-rate quality". The authors point out that proportionately more lower-quality projects have received ROPA funds than other research council grants.

The report suggests that the government needs to prioritize its objectives for ROPA, and that the research councils need to be given more flexibility in running the scheme. Entry requirements need to be adjusted to encourage participation from disciplines that do not strictly fall into the 'industry' category, as well as from younger scientists. But the report does acknowledge that the scheme requires less administration than other comparable grant-awarding mechanisms. E.M.

## BSE transmission data pose dilemma for UK scientists

London. Preliminary research results have confirmed fears that a small proportion of cows infected with bovine spongiform encephalopathy (BSE) can pass the disease on to their offspring, a conclusion that may directly affect the UK government's cattle slaughter programme.

The reported low rate of maternal infections — ten per cent in the offspring of BSE-infected cattle, and an estimated one per cent or more in the national herd — is not expected to affect overall BSE levels. Those "are falling at a rate of 40 per cent a year", according to the Ministry of Agriculture.

An announcement on maternal transmission of BSE had been expected (see *Nature* 381, 724; 1996). But its timing has fuelled further controversy in the United Kingdom and Brussels. Although the 12-member UK Spongiform Encephalopathy Advisory Committee (SEAC) discussed the results at a meeting on 19 July, the official government announcement was made on 1 August, days after parliament closed for the summer.

Opposition politicians are accusing the government of deliberately withholding the results. But Sir John Pattison, chairman of SEAC and vice-provost of University College London, says the time between SEAC's meeting and the announcement was spent reviewing the findings and drafting a statement for government. Pattison says SEAC assessed the data in less than two weeks. "I can't see how we could have acted faster."

This is the second time that SEAC has decided to release results before the scheduled completion of an experiment — or, indeed, its peer-reviewed publication in the scientific literature. The first occasion, when preliminary results suggested an association between the consumption of BSE-infected beef and a new strain of Creutzfeldt-Jakob disease, the human equivalent of BSE, helped to trigger the current beef crisis (see *Nature* 380, 273; 1996).

Francis Anthony, BSE spokesman for the British Veterinary Association, says he was "annoyed" when he heard that preliminary data had been released. "I believe there is a case for the scientists [conducting the experiments] to dig their heels in and say: 'we'll give the results when we're ready', otherwise they could be accused of doing bad science."

But Anthony acknowledges SEAC's dilemma. It had to act quickly in the interests of health, while ensuring the accuracy of the research being publicized, "particularly as I have been urging the government since 1988 to outlaw breeding from the offspring of BSE-infected cattle".

Sir Richard Southwood says the decision to release early data is justified on this occasion. Southwood, professor of zoology at the

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**Making the link: scientists faced a dilemma over the release of data before peer review.**

University of Oxford, was the chairman in 1988 of the government's first advisory committee on BSE, whose recommendations led to the setting up of the maternal transmission experiments. "I know this is a thorny problem," he says. "But this is an important matter. In an ideal world we could wait. But science cannot operate in a vacuum from political, economic or social considerations."

Pattison says the results were released after SEAC members had assured themselves that waiting for further analysis would not have altered the thrust of the findings. "We couldn't just sit on this, and felt we had to tell the government. We knew that a culling policy would need to take account of maternal transmission," he says.

Pattison adds that, if SEAC had waited until the end of the experiments, it would have been accused of endangering public safety. But he acknowledges the dangers of creating an expectation among the public and politicians of the automatic early release of research results, and hopes it will not happen with further BSE-related experiments. "Peer review is absolutely critical; it is the essence of how we operate."

The latest findings come from a seven-year project begun in 1989 at the government's Central Veterinary Laboratory in Weybridge, Surrey. The research compared rates of BSE infection in the offspring of two groups of cattle; analysis of the results has been complicated by the fact that, at an early stage in the experiments, many calves were fed with potentially infected feedstuff.

Of 333 calves born to mothers with BSE, 273 had died by 14 July. Initial histological examination showed that 42 suffered from BSE. An equivalent group was the offspring of BSE-free cattle. Thirteen of these animals had developed the disease. A comprehensive analysis of all the cases would have taken another year.

**Ehsan Masood**

## Citation score boosts Australian institute over research funds

**Sydney.** A comparison of the research performance of Australian universities has confirmed the leading role of the Australian National University (ANU) and its major research arm, the Institute of Advanced Studies (IAS) in Canberra. This has boosted the institute's efforts to fend off greater control over its research agenda by the Australian Research Council (ARC).

The conclusion is based on bibliometric analysis of papers published by all Australian universities between 1981 and 1994. The analysis, by the Institute for Scientific Information (ISI) in Philadelphia, Pennsylvania, is reported in the latest issue of ISI's *Science Watch*. It comes at a time when funding pressures are forcing Australian universities to become more competitive.

The IAS/ANU won first place across major disciplines on two measures: 'impact' — average citations per paper — and total citations. According to the analysis, the two institutions had particular strengths in physical sciences, including chemistry, geosciences, engineering and astrophysics. The IAS has seven postgraduate research schools.

The University of Melbourne came a close second overall, dominating the life sciences by leading both rankings in immunology, molecular biology, microbiology, pharmacology, neuroscience, and biology and biochemistry. The University of Sydney took third place as it "showed depth in the total-citations rankings, appearing among the top three in 14 fields".

The ISI ranking will bolster the ANU's defence against recommendations by the ARC that the research council should have greater influence over the university's block funding and research agenda (see *Nature* **382**, 3; 1996). Sue Serjeantson, IAS director, welcomes the independent score as "confirming" the finding of an earlier review of the institute's research activities. "The bibliometric data underpin, but do not replace, the opinion of the 1995 peer reviewers who said no other Australian institution can match our high standards of performance."

The ANU has been trying to discredit the ARC's report, and to put its case direct to the education minister, Senator Amanda Vanstone. Many of the IAS assessors have written to John Howard, the new prime minister, warning that to disregard the recommendations of the ANU's own review would damage the institute's standing.

Meanwhile Max Brennan, the chairman of the ARC, has criticized the ANU for "continuing its media campaign". He says: "It is inappropriate to comment on allegations by representatives and staff of the university while the government is still considering the ARC's advice." **Peter Pockley**

## US companies accused of 'dumping' waste cargoes

**Hong Kong.** China has turned away a shipment of 200 tonnes of plastics waste from the United States intended for recycling, following the tightening of regulations to comply with a United Nations (UN) treaty on waste exports. Further shipments could be returned to other countries.

The shipment of plastics waste is now stuck in Hong Kong harbour. Chinese officials say there are shipments of scrap metal awaiting return to the United States, Japan, the United Kingdom and other European countries.

The United States and eastern European countries are already talking to Chinese officials about rejected shipments of medical and allegedly radioactive metal waste. William Chen Ping, a Chinese-American businessman, has been in police custody in Shanghai since 3 June for allegedly importing waste illegally.

The plastics shipment has been returned to the Hong Kong trader that negotiated the deal. The waste includes plastic bags from US supermarket chains, and was rejected by Fujian provincial authorities in May because it was mixed with domestic refuse, including rotting food. Hong Kong has so far been unable to return the waste to the United States.

In recent years, China has imported large amounts of plastic, scrap metal, radioactive and medical waste, chiefly from the United States. There have been reports from China of large dumps of foreign waste being created on city outskirts, and of recycling factories in which molten plastic is stirred in open vats without safety controls.

In April, the government passed a law banning the import of waste for disposal, and restricting imports for recycling to a shortlist under a tight permit system controlled by the National Environmental Protection Agency.

A high-profile campaign in China's state-run newspapers has pinpointed the United States as the chief culprit for "using China as a dumping ground". Strong public criticism has been stirred up by shipments of allegedly highly radioactive iron and steel sent from Houston, Texas, to Tianjin, and a load of medical waste allegedly found dumped outside Beijing. But the United States has not been the only culprit; there have also been radioactive metal shipments from Kazakhstan and Kyrgyzstan.

Embarrassed officials at the US supermarket chains from which the plastic originated, including Winn-Dixie, of Florida, say that they had been under the impression

that the bags, returned by customers, were recycled into garden furniture by Trex, a Mobil subsidiary based in Virginia.

Hong Kong's environmental authorities have refused to accept the returned waste, and say the United States has a moral obligation to take it back. But the US exporter, ICP Industries, says the Hong Kong firm knew the purchase was "dirty", and denies any responsibility for problems with China.

Ironically, the situation has arisen partly because China has incorporated a UN convention into its law faster than either Hong Kong or the United States. Under the Basle Convention on Control of Transboundary Movement of Hazardous

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**All at sea? The *Min Hai 451* has been refused permission to land its shipment of plastics waste in China.**

Wastes and their Disposal, signed in 1989, all exported waste must have an export permit, as well as import approval from its destination.

The United States, despite being a signatory of the convention, has not yet ratified it. The US consulate in Hong Kong has bowed to pressure from the colony's government and offered to help find a company in the United States willing to re-import the plastic. The US Environmental Protection Agency has also been recruited to alert US companies to the change in Chinese law.

Meanwhile officials in Shenzhen, a Chinese town close to the border with Hong Kong, say they will return to the colony another nine loads of scrap metal rejected for recycling because of contamination with rubbish — including medical waste.

Hong Kong officials, alarmed at the idea that the colony might be "deluged" with rubbish, have urged the Chinese authorities to send unwanted shipments direct to the country of origin rather than to the colony, which is involved only as a 'transshipment' port.

Mike Stokoe, Hong Kong's director of environmental protection, says that the colony's laws are to be amended by the end of the year to incorporate the Basle Convention. **Lis Tacey**

# Row over plan to treat plants as 'pesticides'

Washington. Eleven scientific societies have criticized proposals by the US Environmental Protection Agency (EPA) to expand its regulatory authority over pesticides to include plants genetically engineered to produce toxic substances.

The EPA published its proposed policy in November 1994, and expects to produce a final version this winter. It defines a "plant pesticide" as "a pesticidal substance produced in a living plant and the genetic material necessary for [its] production". Under the regulation, manufacturers of plant pesticides would be required to apply for EPA permission for late-stage development and marketing.

But the societies that are protesting — including the American Institute of Biological Sciences and the American Society for Microbiology — have issued a report calling the EPA's policy "scientifically indefensible" for proposing to regulate genetically-engineered plants under a law intended to monitor externally applied chemical pesticides.

"Genes and the substances encoded by them" are not pesticides, says the report, delivered to Carol Browner, the administrator of the EPA, last week. The authors say that defining them as such would hurt public confidence in food safety, damage US exports, and impose an onerous regulatory burden on small biotechnology companies.

As a result, they claim, small companies and academic researchers would be dis-

couraged from developing genetically-engineered pest-resistant plants. "It's like putting a skull and crossbones on these biotech products," complains James Thornton of Demeter Biotechnologies Ltd, a small North Carolina company that designs genes for disease resistance in plants.

But major industry groups, concerned about the public acceptability of their products, have joined public advocacy groups in opposing such conclusions. "Just because a substance is in a plant doesn't mean that it's safe," says Lynn Goldman, EPA's assistant administrator for prevention, pesticides and toxic substances.

Rebecca Goldberg, a senior scientist at the Environmental Defense Fund, accuses the societies of having a "double standard" for biotechnology, expecting generous

research funding, but avoiding oversight.

The Biotechnology Industry Association (BIO) has voiced its support of the EPA proposal, but has urged the agency to narrow the definition of plant pesticide to include only active pesticidal agents, and not the genetic material that produced them.

A spokesman says that the complaining scientists have misread the Federal Insecticide, Fungicide and Rodenticide Act. "The pesticide law gives EPA very broad authorities to regulate pesticides," says Alan Goldhammer, BIO's director of technical affairs. "It doesn't say that they can't regulate plants."

Other industry groups and companies stress the importance to consumers of regulations such as the one proposed. "A strong government oversight system is important to public confidence," says Loren Wassell, spokesman for Monsanto, the agricultural and chemicals company.

Monsanto was the first company to gain EPA approval for commercial marketing of a plant genetically engineered for pest protection. Its NewLeaf potato, first marketed in 1995, was protected against the Colorado potato beetle by a gene derived from *Bacillus thuringiensis*, a soil bacterium.

Companies may at present voluntarily submit plants with genetically engineered traits for pest resistance for EPA approval, as did Monsanto. If the new policy is adopted, such approval would become mandatory.

Meredith Wadman



## Public spending curbs threaten Israeli research projects

**Jerusalem.** New research projects are likely to see their funding reduced as a result of public spending cuts that form part of an anti-inflation programme approved last month by Israel's cabinet.

The full package of cuts — which was called for by the new prime minister, Benjamin Netanyahu — has still to be approved by the Knesset, Israel's parliament. But government officials are already considering how to absorb them with the least damage to research programmes.

Hardest hit is likely to be the Ministry of Science, which funds primarily medium- and long-term research. The cabinet has cancelled a decision by the previous government to increase the ministry's budget, and has reduced its budget for next year by US\$3.5 million or 7 per cent.

Ministry officials are concerned that the government's commitment to scientific and technological advancement will lose its credibility. "In terms of the message it sends, we'd be happier if the budget were at least growing in a symbolic way," says Moshe Mishal, the ministry's deputy director-general for planning and oversight. "A

\$3.5-million slash cuts into the flesh."

About a third of the ministry's budget (which totals \$47 million this year) goes to special funds and programmes, and another third to joint scientific programmes with other countries. A technological research and development treaty between Israel and the European Union, ratified by the Israeli cabinet last week, requires an annual Israeli contribution of \$30 million, part of which will come from the ministry's budget.

Ze'ev Binyamin (Benny) Begin, the minister of science — a geologist by training — points out that the funding provided by his ministry is only a small portion of the \$1 billion that government and industry spend on research and development each year. Nevertheless, the areas that it funds are of importance to Israel's economic and technological advancement.

One institution seriously concerned about the cuts is the Technion — Israel Institute of Technology — where science ministry grants are an important component of the research budget. According to Felix Naggar, head of Technion's project advancement department, the ministry signed

research contracts worth \$5.5 million with his institution last year.

Arnon Bentur, Technion's vice-president for research, says it is too early to say what the effects of the cuts will be. But he is also concerned about expected cuts at the Ministry of Industry and Commerce, whose research budget is likely to be reduced by 4.5 per cent, or \$17 million.

The industry ministry awards grants to companies and academic scientists for applied research and development. Although the Technion received only \$900,000 directly from the ministry in 1995, it benefits in a much larger way indirectly. Many research projects commissioned by Israeli industries or performed jointly by industry and Technion scientists are funded by the ministry.

"In many ways the industry ministry cuts will be harder on us," Bentur explains, because the amounts are larger and because "we have invested in a lot of infrastructure to fulfil the [ministry's] needs." The danger, he says, is that, "after we have bent over backwards to help the ministry, we will be left with infrastructure that won't be used".

Haim Watzman



# German institute renews east-west unease

**Munich.** East-west tensions have erupted in Berlin over a decision to move a molecular pharmacology research institute from decrepit buildings on the eastern edge of the city to a purpose-built home at the Max Delbrück Centre, a national research centre for molecular medicine.

The decision, announced two weeks ago, has been welcomed by the staff of the Institute for Molecular Pharmacology, known as the FMP. After a rocky start, the future of the institute is now looking secure following the appointment this month of its first permanent director, five years after its foundation.

But the decision has been strongly criticized by west Berlin's nearly bankrupt Free University, which, keen to boost its scientific credentials, had fought hard to host the institute.

The FMP was founded as a successor to a former research institute of East Germany's Academy of Sciences on the recommendation of Germany's science council, the *Wissenschaftsrat*, which evaluated all the academy's research institutes after reunification. The science council had been particularly impressed by the strength of the former institute's peptide synthesis unit.

But the new institute has had great difficulty in finding a permanent director. Three west German scientists were appointed, only to withdraw before accepting the position permanently. A crucial factor in each case was the frustration of dealing with the complicated politics of a city facing the financial burden of rebuilding the east. None of the candidates was prepared to give up a more certain career in the west.

Four years ago, the FMP's scientific advisory committee decided that, to overcome its isolation from the academic community, the institute should relocate to the Berlin

Buch science area in the east of the city, and link up with the Max Delbrück Centre, also founded after reunification. The federal and Berlin research ministries agreed in principle to split the costs of a new building.

Since then, however, Berlin's financial situation has deteriorated considerably (see box, below left), and tensions between 'wessies' and 'ossies' have increased. The signing of the financial agreement was delayed, and uncertainty has increased for the institute's 150 staff.

The delay was exploited earlier this year by the Free University, which has suffered heavily from Berlin's financial problems, with massive reductions in student numbers and the closure of faculties and departments (see *Nature* 380, 278; 1996).

Seeing the chance to strengthen its science base, it put in a counter-bid to host the institute, including an offer of university chairs to several leading members of the FMP. The attractiveness of this offer threw further doubt on the Buch option, and a political battle began.

The Berlin government was split. Some wanted the Free University's concept to be developed further because of the potential savings, but others felt that as a matter of principle the institute should not be transferred to a western institution.

The federal government opposed the Free University's offer, as it did not want to be seen to be financing what could be mistaken for a university institute. According to the German constitution, universities are exclusively the domain of *Länder* (state) governments.

Last month's final decision to transfer the institute to Buch has done little to ease east-west tensions, even though the FMP's new director, Walter Rosenthal, describes the agreement as "a good choice".

Rosenthal says that he is "glad that the power struggle is over". He is particularly pleased that the institute will remain under one roof, as this would probably not have been possible under the university's plan. But he accepts that for the next few years he will be preoccupied with the political and administrative battles that are likely to continue until the institute is running smoothly.

However, Peter Gaetgens, dean of the science faculty of the Free University, says that he is disappointed by the decision, which he describes as "a wrong one". He

Kai Blnert

IMAGE  
UNAVAILABLE  
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REASONS

**On the move: the Institute for Molecular Pharmacology will soon have a new home.**

says that the Max Delbrück Centre already has enough scientists. By contrast, he argues, German universities have been weakened by increased teaching loads, and Berlin's in particular by devastating budget cuts.

Gaetgens insists that the FMP would have gained considerably from the links that the university was offering. He expresses doubt that the university will now make a significant number of chairs available for FMP staff, as the 50 km separating Buch and the Free University make meaningful interaction unlikely.

But Folk Fabich, head of the Berlin Research Association, *Forschungs Verbund Berlin*, which administers eight research institutes, has little sympathy with the university. He criticizes the three former acting directors for making commitments they were not prepared to meet, and says he is glad the institute is moving to Buch.

Fabich points out that the FMP is not the only institute in Berlin to have suffered from the difficulty of keeping a director. The Ferdinand Braun Institute for High Frequency Technology is also this month appointing its fourth director in five years.

A former director, Peter Russer, who returned to his chair in Munich last year, admits that "it is hard for a westerner to survive in an east German research institute", as there remains a clash of cultures and difficulties in establishing competitive research programmes. **Alison Abbott**

## Berlin's universities face further squeeze

**Munich.** Berlin's science and research budget could face further cuts next year of up to DM160 million (US\$108 million), according to documents leaked from the city's finance ministry. A DM200-million cut was imposed on Berlin's three universities in an emergency budget earlier this year.

If formally approved, the new cuts would almost certainly have to be absorbed entirely by the universities, as the funding of non-university research institutes is fixed by binding agreements with the German federal government.

University spending is a low priority in Berlin, which is verging on bankruptcy. The rumoured cuts represent more than a third of the reductions in the city's whole budget next year, and would fall in

particular on younger staff and students. Professors have tenure, and are not easy to dismiss.

The leaked documents suggest that the universities cannot expect any budget increase before the end of the decade at the earliest. That raises the spectre that one of the city's universities may have to close. Student numbers have already been cut drastically, and many faculties have been merged or closed.

The proposed cuts have been attacked by Peter Radunski, Berlin's research minister, who is to meet Eberhard Diepgen, the city's mayor, this week to defend the universities. A final decision on the 1997 budget will be made next March. **Quirin Schiermeier**



## Environment agency panel to 'steer' research plans

**Washington.** Costel Denson, vice-provost for research at the University of Delaware, is to chair a Board of Scientific Counsellors set up by the research office of the Environmental Protection Agency (EPA) to advise it on its programmes and procedures.

The board, whose 15 members were nominated by laboratory directors of the Office of Research and Development (ORD), meets for the first time on 19 August.

EPA already has a Science Advisory Board, but its main purpose is to evaluate research after it is finished, according to ORD chief Robert Huggett. The new group will advise ORD on its research agenda and will help the office to set up procedures for peer review of outside grants as well as of its own laboratories and personnel.

But it will not duplicate any of the activities of the Science Advisory Board. That way, says Huggett, the group recommending what research the office should be pursuing will not be responsible also for reviewing the products of that research.

In the two years since Huggett arrived at EPA, the ORD has made a high-profile

effort to increase its ties with outside scientists, quadrupling to \$80 million the amount it spends on external, peer-reviewed grants. Although this has earned praise from outside advisory groups and from Congress, the ORD has created a logistical nightmare, says Huggett, in having to handle thousands of new grant proposals.

The National Science Foundation (NSF) has helped EPA to set up review procedures for some of the grants. But Huggett admits that his office is swamped with additional paperwork. One of the new board's first duties will be to help the office to reorganize its peer review procedures.

One solution might be to ask for pre-proposals, says Huggett. Another is to cooperate with other federal agencies on joint solicitations. He has been meeting the NSF, energy and defence departments and other agencies to help identify opportunities for jointly funded research. A joint solicitation on bio-remediation research with the US Navy, energy department and NSF is expected to go out in October.

**Tony Reichhardt**

## Chemistry professor takes over Islamic science organization

**London.** COMSTECH, an intergovernmental organization promoting scientific and technological cooperation within the 50-member Organization of Islamic Countries (OIC), has appointed a new coordinator-general.

Atta ur Rahman, professor of chemistry at the University of Karachi, takes over from M. A. Kazi, science adviser to Pakistan's former military president, General Zia ul Haq. Rahman will remain director of the University of Karachi's H. E. J. Research Institute of Chemistry.

The institute carries out research in natural product chemistry, and claims to produce half of Pakistan's annual output of around 50 PhDs.

Rahman has a high profile in his own country as probably the best known scientist after Abdul Qadeer



Rahman: will seek to boost key disciplines.

Khan, director of the uranium enrichment laboratory at Kahuta. But he is controversial, having recently announced his institute was close to finding a therapy for AIDS.

Despite convening regular conferences and publishing journals, COMSTECH is viewed as having failed to strengthen research institutions and to promote scientific links between OIC member states.

Rahman says one of his priorities will be to strengthen industrial biotechnology, renewable energy and arid agriculture. Another will be to encourage researchers to make better use of international funding sources. His own institute was recently awarded Ffr30 million (US\$6 million) by the French government. He says that COMSTECH's first 13 years can be characterized by "a lack of vision and the naive belief that people will give you money regardless of the quality of your research proposal".

COMSTECH, whose secretariat is based in Islamabad, is one of three OIC science bodies set up in the early 1980s when the organization was awash with Middle Eastern largesse. The other two — the Islamic Scientific, Educational and Cultural Organization, and the Islamic Foundation for Science, Technology and Development — are now less active.

The Pakistan government was keen not to let COMSTECH follow suit and last year gave it a substantial grant for an international conference intended to revive the organization.

Ehsan Masood

## South African minister to quit post

**Cape Town.** South Africa's minister responsible for science and technology, Ben Ngubane, is to leave the national cabinet, in which he also holds responsibility for arts and culture, at the end of the month to take up an (as yet unannounced) portfolio in the KwaZulu-Natal provincial cabinet.

Ngubane's move was announced by Mangosuthu Buthelezi, the leader of the Inkatha Freedom Party (IFP), at the party's annual conference last week. Buthelezi is said to have been unhappy about the good working relationship that Ngubane, a physician, enjoys both with his cabinet colleagues and with foreign governments.

President Nelson Mandela appears not to have been consulted. Nor is he understood to have spoken to Ngubane about it since the announcement. Under the terms of the interim constitution, the party is entitled to three cabinet portfolios. But the state president has the prerogative to decide which they should be.

There is little enthusiasm in government circles for the IFP retaining the arts, culture, science and technology portfolio. This is for two reasons. The first is that the department has felt marginalized as a result of having a minister who is not a member of the

African National Congress, the largest party in the coalition government.

The second is that the most likely IFP incumbent to succeed Ngubane is Lionel Mtshali, the party whip. As former education minister in the KwaZulu homeland government, Mtshali enthusiastically implemented a system of patronage under which all officials and teachers employed by his department had to be IFP members.

One alternative is that Mandela might dissolve the ministry, and allocate another portfolio to the IFP. He could then either place the ministry's diverse functions under deputy president Thabo Mbeki, or divide them between existing ministries. If the latter route were to be chosen, science and technology might be allocated to Alec Irwin, the minister of trade and industry, with arts and culture going to Sibusiso Bengu, minister of education.

Ngubane declined to comment on the move, which, ironically, has come just as the ministry seemed to be making real progress after a frustratingly slow start. But his departure is not expected to delay the white paper on science and technology, due to be presented to cabinet later this month, before he leaves his present post.

**Michael Cherry**

## German physicists oppose cuts to international research labs

**Munich.** The German Physical Society is to lobby the German government over its proposed cuts to international research laboratories such as the European Laboratory for Particle Physics (CERN) and the European Southern Observatory (ESO) (see *Nature* 382, 285; 1996). The society's president, Detlev Schmitz, a particle physicist from the University of Aachen, says that it plans to prepare a document for the research ministry in September that is likely to defend both CERN and ESO.

The budget reductions will have both national and international implications. Schmitz says that cuts at CERN and ESO will have an immediate effect on physics research projects carried out in German universities. Riccardo Giaconni, director of ESO, says that if the cuts are approved, they could delay ESO's Very Large Telescope beyond its current completion date of 2000, and thus force up its costs. □

## Nuclear plant voted out

**Tokyo.** In Japan's first local referendum on nuclear power, the residents of a small town on the coast of the Japan Sea have voted against the construction of a nuclear plant in their town, setting a precedent that is likely to increase the difficulties of the national government in pursuing its plans to expand nuclear power.

There was a large turnout — over 88 per cent — for the plebiscite in Maki in Niigata Prefecture, a town with fewer than 24,000 eligible voters. Nearly 12,500 voted against the nuclear plant. After the vote, Takaaki Sasaguchi, the mayor of Maki, who proposed the plebiscite in his election campaign in January, repeated his pledge to bar the sale of land on which Tohoku Electric Power Company has for years been proposing to build the nuclear plant. □

## Radiological limit 'misguided'

**London.** Britain's National Radiological Protection Board (NRPB) has described as "misguided" and "fraught with danger" attempts to propose a minimum threshold for radiation. In its annual report, published last week, Roger Clarke, director of NRPB, reiterated the board's opinion that there is no such thing as a radiation dose that carries zero risk.

"The pursuit of a threshold is an attempt at a quick fix for problems that require a more deliberate approach," writes Clarke. "The real issue to be decided is the acceptability of risk — where does society set levels of unacceptability or triviality of risk". He suggested it would be more appropriate to assume a progressive increase in risk with increasing dose. □

## Impasse on N-waste storage

**Washington.** The US Senate last week voted 63–37 in favour of a bill that would allow spent nuclear fuel to be stored at Yucca Mountain, Nevada, on an interim basis, until a decision is reached to build a permanent underground repository there. But President Bill Clinton has said that he will veto the bill, and with no sign that supporters can muster the 67 senators needed to override a veto, the spent fuel will be staying just where it is — in temporary storage at 80 nuclear power stations around the country. □

## 'Protect Internet' for UK scientists

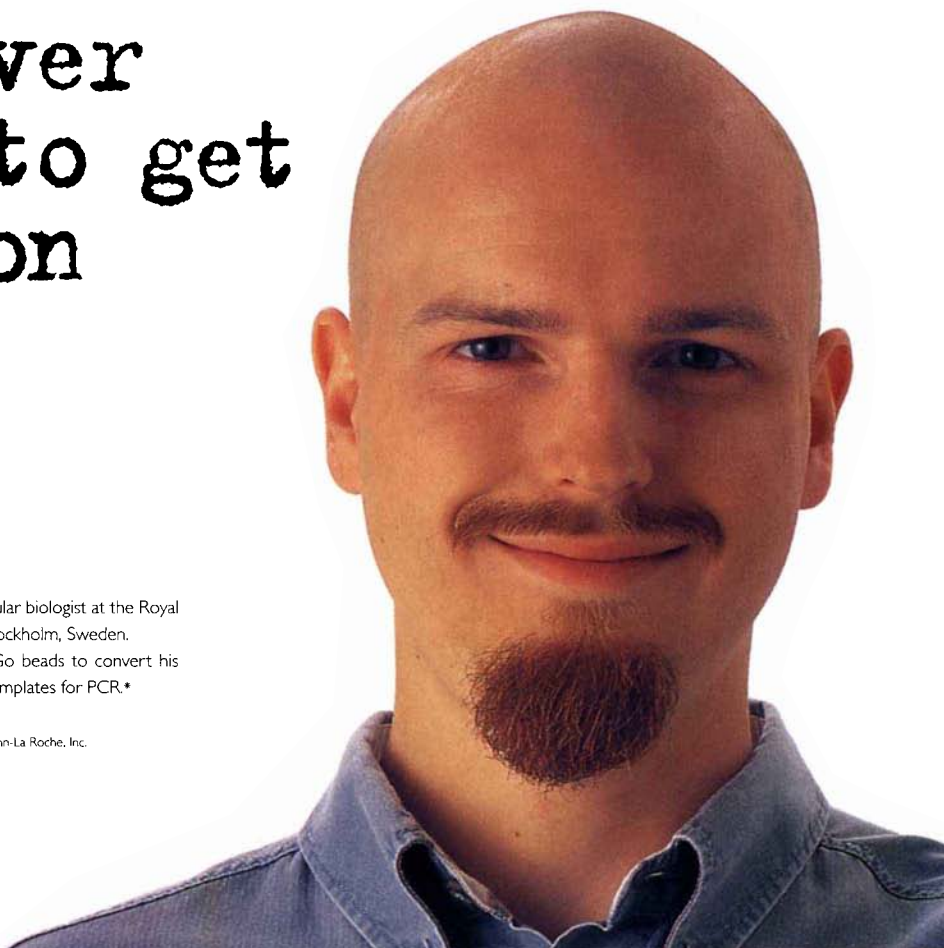
**London.** The House of Lords has called for moves to protect Internet bandwidth for British academic researchers, possibly by creating a US-style high-speed computer network dedicated to research (see *Nature* 380, 93; 1996). The recommendation is contained in a report on UK information technology policy, published by the Lords' select committee on science and technology.

# Patrik never fails to get a reaction

Patrik Samuelson is a molecular biologist at the Royal Institute of Technology in Stockholm, Sweden.

Patrik uses Ready-To-Go beads to convert his RNA samples into cDNA templates for PCR.\*

\* PCR is a patented process of Hoffmann-La Roche, Inc.





Other suggestions include the setting-up of an Information Society Task Force — also similar to one in the United States — to act as an information technology 'think-tank' to the government and circulate views on the development of the UK 'Information Society'. The report also asks the government to urge the United States to relax restrictions on the export of encryption technology. □

## Launch vehicle crashes out

**Washington.** A prototype experimental launch vehicle, originally developed for the US military but now operated by the National Aeronautics and Space Administration (NASA), crashed in the New Mexico desert last week, bringing a premature end to a \$50-million effort to test-fly a rocket that could take off and land vertically.

The 'Clipper Graham' tipped over and caught fire after its landing gear failed to deploy properly. The vehicle had flown successfully three times, and was due for one more test this summer. NASA will continue with a more ambitious programme to develop the X-33 rocket, which takes off vertically but lands like an aeroplane. □

## Immunologist is reinstated

**Washington.** Tufts University has reinstated Thereza Imanishi-Kari, whose academic career had been derailed by accusations of scientific misconduct from which she was recently exonerated. The university's School of Medicine last week reinstated the immunologist as an assistant professor of pathology, a tenure track position, retroactive to 1 July.

In June, an appeals board of the US Department of Health and Human Services (DHHS) cleared Imanishi-Kari of accusations of scientific wrongdoing brought by a junior colleague, ending a 10-year controversy (see *Nature* 381, 789; 1996) "It's wonderful," Imanishi-Kari said from her laboratory, where she has continued her work using private research funds. "Proper due process did take place." □

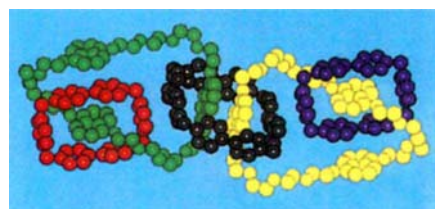
## Health law limits genetic data use

**Washington.** A health insurance bill passed separately by both bodies of the US Congress last week forbids insurers from denying coverage to workers who lose or change jobs based on 'genetic information' — the first time that Congress has legislated in the rapidly evolving area of genetic discrimination.

The vote on genetic information "is a win for the people concerned about this issue", said Lyle Dennis, director of the Genome Action Coalition, a Washington-based advocacy group. The law allows insurers on a one-time basis to refuse to cover pre-existing medical conditions for up to one year. But it explicitly excludes genetic information from the definition of a pre-existing condition, unless a diagnosis consistent with the information has been made. □

## 'Olympic' molecule takes to track

London. **British scientists have created a synthetic molecule (right) that mimics the five Olympic rings, but which is smaller than a grain of salt. The molecule**



**has been named 'Olympiadane' and is expected to pave the way for the storage of information on chains of molecules, leading eventually to the creation of 'molecular computers'.**

But "it could be a couple of decades before applications become a reality," says David Williams, professor of structural chemistry at Imperial College London. The Olympiadane itself is the result of a 20-year collaboration between Williams and Fraser Stoddart, professor of organic chemistry at the University of Birmingham. □

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# Is the Pope the Pope?

SIR — Beck-Bornholdt and Dubben (*Nature* **381**, 730; 1996) describe a common misinterpretation of the  $P$ -value of a classical statistical test using the following example. The chance that a randomly chosen human being is the Pope is about 1 in 6 billion. John Paul II is the Pope. What are the chances that John Paul II is human? By analogy to syllogistic reasoning, Beck-Bornholdt and Dubben suggest 1 in 6 billion but point out that this is “obviously not sensible”.

It certainly isn't. The probability of data given a hypothesis,  $P(D|H)$ , is not the same as the probability of the hypothesis given the data,  $P(H|D)$ . This is an elementary error regardless of one's preferred statistical approach. Bayesian statistical inference, which includes syllogistic reasoning as a special case, is particularly well-suited for avoiding this sort of mistake (H. Jeffreys, *Theory of Probability*, Oxford University Press, 1939).

A similar pitfall is the infamous ‘prosecutor's fallacy’, in which a probability that a DNA fingerprint match would occur in someone other than the true criminal —  $P(\text{match}|\text{innocent})$  — is used incorrectly as the probability that a suspect is innocent —  $P(\text{innocent}|\text{match})$ . In a city of ten million people, a one-in-a-million DNA fingerprint match will give ten other people the same fingerprint as the true criminal. In the absence of other evidence, the odds that the suspect is innocent are better than 90%, not one in a million.

Let  $H$  represent the class (hypothesis) of humanness,  $A$  represent the class (hypothesis) of alienness, and  $J$  represent the observation (data) that a randomly chosen individual is Pope John Paul II. Bayes's theorem tells us how to infer the probability that the Pope is human,  $P(H|J)$ :

$$P(H|J) = \frac{P(J|H)P(H)}{P(J|H)P(H) + P(J|A)P(A)}$$

Thus, to infer the probability that the Pope is human,  $P(H|J)$ , we have to have two more numbers in addition to the probability  $P(J|H)$  of drawing the Pope at random from the class of humans:

(1)  $P(J|A)$ , the probability of choosing the Pope at random from the class of aliens.

(2)  $P(A)$ , the *a priori* probability that a randomly chosen individual is an alien instead of a human.

$P(H)$  is just  $1 - P(A)$ , if only these two hypotheses are considered.

Presumably the probability  $P(J|A)$  of choosing the Pope from the class of aliens is infinitesimal. The prior probability of choosing an alien as opposed to a human,  $P(A)$ , is also expected to be quite small, except perhaps near secret US Air Force bases. As either  $P(J|A)$  or  $P(A)$  approaches zero, the probability that the Pope is human approaches one.

It is a shame that Bayesian methods are not part of all introductory statistics classes. In this case, they quickly reassure us that the Pope is (probably) not an alien.

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SIR — In their syllogistic reasoning to present a papal paradox, surely Beck-Bornholdt and Dubben's sequence is incorrect. It should be as follows: (1) If an individual is human s/he is probably not the Pope (premise); (2) John Paul II is human (premise); (3) therefore John Paul II is probably not the Pope (conclusion).

In fact, of course, he is the Pope, but probability allows for this possibility (it was just extremely unlikely) and the logical progression holds. Their sequence made the classic mistake of assuming that “if  $A$ , then not  $B$ ” implies “if  $B$ , then not  $A$ ”, which can be disproved by many examples more trivial than the papal paradox. Indeed, if applied to their first example, it would imply that all mortals are human, a hypothesis easily disproved by considering any other living being.

If the logical sequence they describe for statistical hypothesis testing is actually the one in use, then it too makes the same mistake, and is therefore in error, but not for the reason they suggest. I think the conclusion in the sequence should be “(3) therefore the null hypothesis is PROBABLY wrong”, with probability theory providing the degree of uncertainty, leading to conclusions such as “it is extremely unlikely (5% probability) that this result arose by chance” — but I'm probably wrong!

**Stephen P. Gosden**

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SIR — The argument of Beck-Bornholdt and Dubben can be paraphrased as follows. We have a human-looking organism before us and wish to test the hypothesis that it is in fact human. We know that if it is human, it is very unlikely to be the Pope. We find out that it is indeed John Paul II, and have to conclude that it is unlikely to be human. In claiming that this conclusion (and with it, statistical hypothesis testing) is absurd, Beck-Bornholdt and Dubben not only contradict one of their own assumptions but also describe bad scientific practice.

We use statistics to test hypotheses because we know that the same set of data

could arise whether or not a given hypothesis is true. In making the comparison with statistical hypothesis testing, Beck-Bornholdt and Dubben assume at the outset that a non-human could be the Pope. They cannot therefore claim that the conclusion of their argument is ridiculous, merely that it is factually wrong.

Furthermore, this incorrect conclusion arises only if the randomly picked human-looking organism happens to be the Pope. On the many other occasions when we perform the experiment, we will quite reasonably fail to reject the hypothesis that our subject is human. The same applies to statistical reasoning: we will occasionally observe highly improbable data, and incorrectly reject the null hypothesis.

In their statement of their argument, Beck-Bornholdt and Dubben have implicitly allowed themselves the luxury of observing subjects until they find one that is the Pope. This procedure is equivalent to a scientist, anxious to reject a null hypothesis, repeating an experiment until a suitable result turns up.

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SIR — Beck-Bornholdt and Dubben have shown at most that the Pope is not a random sample. Their ‘syllogism’:

Premise: “If... we randomly pick a human being, the probability that it is the Holy Father is extremely low...”

Conclusion: “Therefore, if an individual is human, it is probably not the Pope.”

A syllogism states that an attribute shared by all members of a class is possessed by each member. Their conclusion ignores the fact that the attribute of probable nonidentity with the Pope was limited explicitly to the class of randomly chosen human beings. If the sampling method is not part of the class specification, why not just give as the premise “The Pope is, with high probability, not the Holy Father,” thus suggesting an even wittier title for the published letter?

Statistical inference about membership in a hypothesized distribution applies to data randomly sampled from the population of inference. The failure of inference here results from no philosophical paradox about probability, but only from verbal shiftiness about the population and the sampling method.

**James C. Nelson**

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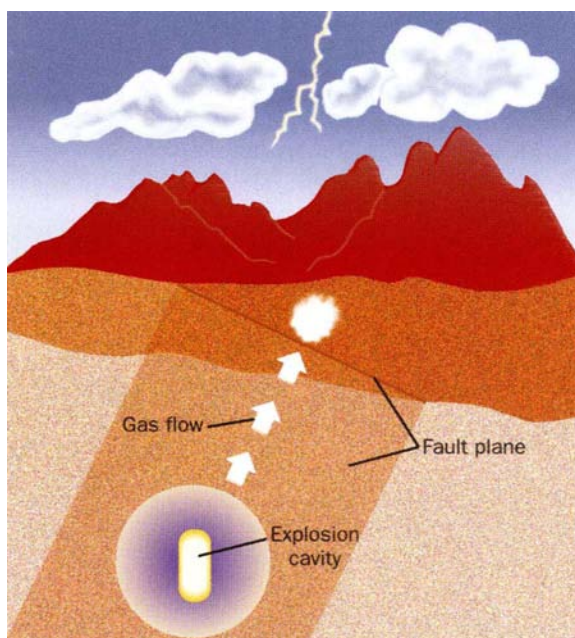
# Sniffing out clandestine tests

Lars-Erik De Geer

**An experiment at the Nevada Test Site demonstrates that there are good prospects for catching a nuclear test ban violator by detecting radioactive noble gases leaking through geological faults.**

IMAGINE that, under a comprehensive test ban where no nuclear explosions are allowed, some evidence comes along indicating that an explosion has nonetheless taken place. The International Monitoring System (IMS), set up to verify compliance with the test ban, has reacted, and its seismic and/or radionuclide arms show that there has been a very suspicious event in a certain country, signatory of the treaty. The IMS cannot define the exact latitude and longitude of the suspected event, but it can give a location uncertainty ellipse of, it is hoped, less than 1,000 square kilometres. That is a large area, comparable to the size of the biggest cities in the world, so finding the exact point of interest during an on-site inspection is not necessarily an easy task.

One of the potentially most powerful and least intrusive on-site inspection search techniques prescribed in the protocol of the planned treaty (see box) is scanning for radionuclides in the air just above or below the ground. In most cases, the detection of radioactive fission products and/or activation products in this way would be very strong evidence that a nuclear explosion had taken place. It would probably also help reduce the



A cross-section of the non-proliferation experiment, where gas leaks were found months after a deeply buried 1-kt simulated nuclear explosion. The gas travelled mostly along faults and fractures, sucked out by atmospheric depressions.

search area substantially, and together with other techniques could define the exact ground zero.

But if a violator chooses to test deep in the ground to evade detection, will any radionuclides make it to the surface?

In this issue, C. R. Carrigan *et al.* (*Nature* **382**, 528–531; 1996) report the results of what was called the non-proliferation experiment. This experiment shows convincingly that a violator runs a great risk of being caught during an on-site inspection via detection of radioactive noble gases, notably xenon-133 and argon-37, sucked through geological faults from the underground cavity by atmospheric depressions.

This does not prove that under all circumstances xenon and argon isotopes will find a path to the atmosphere, but it certainly highlights a problem and a great source of uncertainty for the cheat. Trying to steer clear of this risk might be forbiddingly expensive, or it might force the test point into an environment that is more susceptible to detection by some other on-site inspection technique.

On 22 September 1993, more than 1,000 tonnes of chemical explosives were set off in a mined cavity 400 metres below the Rainier Mesa at the Nevada Test Site in New Mexico, United States. That was two to three times deeper than common practice for a one-kilotonne underground nuclear test. Two gas bottles were placed close to the explosives, containing about 0.2 kg of helium-3 and 50 kg of sulphur

## A comprehensive test ban treaty

THIS year there are great expectations that after decades of stumbling negotiations, talks and initiatives, a full and complete ban on all kinds of nuclear explosions will be signed within the Conference of Disarmament in Geneva. Its target date was 28 June, the last day of the conference's second session this year, but owing to disagreements on its scope, on which countries need to sign before entry into force, and on the rules for initiating an on-site inspection, the negotiations had to be postponed to the third session, which started on Monday last week. At present, India alone is blocking the test ban by holding it hostage to total nuclear disarmament — a second-best solution held hostage to

the best. This is ironic, as India was the first country to introduce the test ban question to the world, back in 1954.

Within the treaty structure two major components for verifying compliance are foreseen: an International Monitoring System (IMS) and an on-site inspection regime. The IMS is composed of four global networks that will continuously check for suspicious seismic signals in the ground, hydroacoustic sound in the high seas and infrasound and radioactive signatures in the atmosphere. When an event is detected that is still suspected of being a nuclear explosion after initial cross-checking, requests for clarification and other inquiries, an on-site inspection team might be launched by

the Executive Council to look for corroborative and conclusive evidence.

The goal of the IMS is that it should be capable of defining a suspected area of less than 1,000 square kilometres. A number of techniques have been discussed for different stages of the ensuing on-site inspection, including quite obvious things like visual observations from the air, spectral imaging, a search for seismic aftershocks and sniffing for radionuclides, to more esoteric techniques for detecting cavities, like magnetic- and gravitational-field mapping and resonance seismometry. For final proof, one will always have to drill for samples of radioactive glassified rock in the cavity.

L.-E. De G.

hexafluoride. These non-radioactive gases are suitable as tracers because of their very low presence in the environment. Nearly 200 samples were then taken in the area over the next one and a half years, with a strategy prejudiced by the thinking that the gases should first appear near geological faults and during times of low atmospheric pressure (see figure).

The fact that radioactive gases can be sucked along faults and fissures from an underground nuclear test point is known from past testing experience. The authors, however, do not cite anything of that. Perhaps leaks from test sites are too sensitive a subject for anyone to talk about, even in the case of noble gases with very little impact on man.

The educated guesses were, however, nicely confirmed by the non-proliferation experiment. The first positive finding came during the first real post-shot sampling campaign: during a deep depression in November, nearly 50 days after the explosion, sulphur hexafluoride was detected in fractures along a nearby fault at concentrations of more than 100 times background. Two hundred times background was seen the following spring, and less during the subsequent autumn. All detections were during atmospheric depressions, and all but one were near faults or surface fissures.

Helium-3 appeared much later than sulphur hexafluoride.

The first hit came in October of the second year, about 375 days after the shot, when the mass spectrometer showed a value more than two standard deviations above background. Higher values were then seen in November on a fault closer to the explosion.

At first glance it is surprising that helium-3, a smaller and much more diffusive molecule than sulphur hexafluoride, does not arrive at the surface first, but lags almost a year behind. The reason is simple and thought-provoking. Gas transport is by no means dominated by diffusion — that would take tens to hundreds of years. Instead, it occurs at a rate an order of magnitude higher, along pre-existing faults and fractures. And easy diffusion now works against the flow: helium diffuses eagerly into the porous walls of the fractures, and is accordingly delayed.

Carrigan *et al.* applied a theoretical model to this process. By adjusting the single free parameter, the width of the fractures, to 1.25 mm to fit the helium-3 data, the sulphur hexafluoride behaviour was closely reproduced. This suggests that the model should also work for other gases such as the above-mentioned noble gases xenon-133 and argon-37.

The model forecasts that had the explosion been nuclear instead of chemical on that day in Nevada, xenon-133 should

have been detectable 50 days post-shot and argon-37 another 30 days after that. Radioactive decay has been taken into account, with the two half-lives of 5.3 and 34.8 days, respectively. These calculations are robust, as they assume conservative values both for detection limits and for production yields. The detection limit for xenon-133, for example, is assumed to be a million times higher than attainable with current techniques (which are improving rapidly). That does not necessarily mean that it could be detected earlier, because the barometric low after 50 days would have been as much a decisive factor for the xenon as it was for the sulphur hexafluoride. It implies, however, that an on-site inspection has a good chance of finding conclusive evidence for a 'well-contained' clandestine nuclear explosion for several months afterwards.

The non-proliferation experiment has shown that looking for radioactive noble gases in the defined search area of an on-site inspection is a very powerful technique. An on-site inspection should, of course, be launched as soon as possible, but even if delayed for a month or two the cheat should certainly not feel safe. □

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## TRANSCRIPTION

# A fate worse than death

Craig B. Thompson

DURING the development of many, if not all, complex organisms, specific cells are marked out for elimination<sup>1,2</sup> in a process known as programmed cell death, or apoptosis, a form of cell suicide. For example, during the development of the hermaphrodite nematode worm *Caenorhabditis elegans*, 131 of the 1,090 cells produced are genetically destined to die. *Drosophila* embryos without the necessary genes to execute this death programme do not survive. In vertebrates, failure to delete malformed or potentially autoreactive immune cells during development can eventually lead to autoimmunity or leukaemia. So too much or too little cell death threatens the whole organism.

Although several evolutionarily conserved genes that execute a common cell-death pathway have been identified<sup>3</sup>, we still know relatively little about the genes involved in targeting specific cells for death during development. Two groups working in seemingly unrelated fields have now identified a family of transcription factors that seem to be important for determining which cells will die. On page 545 of this issue<sup>4</sup>, Metzstein *et al.* describe

the cloning of a gene, *ces-2*, that orchestrates the death of two cells in the posterior pharynx of developing *C. elegans*. This gene turns out to be a member of a subfamily that encodes a transcription factor of the bZIP type, so called because it contains a structural motif known as a basic 'leucine-zipper'.

Another transcription factor in this subfamily, hepatic leukaemia factor (HLF), was discovered because of its localization at the site of a chromosomal translocation that is a feature of acute lymphocytic leukaemia. And on page 541 of this issue<sup>5</sup>, Inaba *et al.* show that the chimaeric protein created as a result of this translocation, E2A-HLF, prevents cells from responding to apoptotic stimuli.

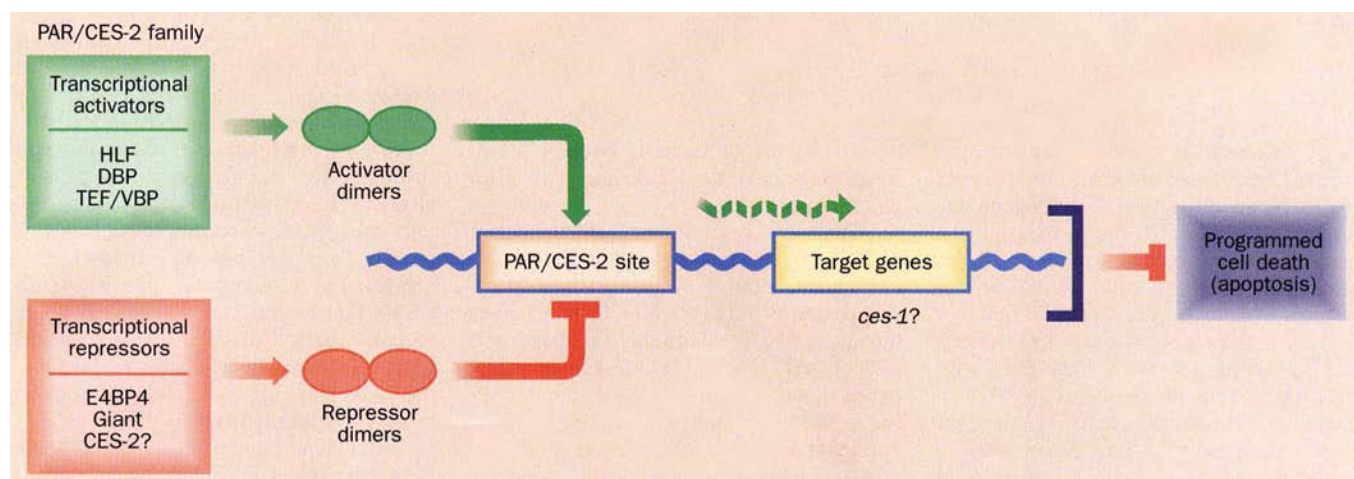
The *ces-2* gene was originally discovered during a screen for *C. elegans* mutants that had altered patterns of expression of the neurotransmitter serotonin. Loss-of-function mutations in *ces-2* result in the appearance of two additional serotonin-containing neurons in the pharynx of developing worms<sup>6</sup>. These two cells are the sisters of the nerve cells known as NSM neurons. Normally during development,

these two cells would be programmed to die, but in *ces-2*-mutant animals the NSM sister cells live. *ces-2* is unique in that its inactivation appears to affect the survival of only the NSM sister cells.

In contrast, mutations in genes that are involved in the execution of the central cell-death pathway have much wider effects on developmental cell death. For example, in animals defective in the protease CED-3, a lethal effector of apoptosis, all 131 of the cells that normally die survive. It is thought that *ces-2* promotes the death of the NSM sister cells by inhibiting a second gene, *ces-1*, which seems, either directly or indirectly, to inhibit the activity of death genes in specific cells; in animals carrying a gain-of-function mutation in *ces-1*, neither of the two NSM sister cells nor the sisters of two additional pharyngeal neurons undergo programmed cell death.

The subfamily of bZIP transcription factors, to which *ces-2* appears to belong on the grounds of its similar sequence<sup>4</sup>, includes three activators of transcription in mammals, namely HLF and two other proteins known as albumin D-element binding protein (DBP) and thyrotroph embryonic factor/vitellogenin gene binding protein (TEF/VBP), as well as two transcriptional repressors, encoded by the *Drosophila* gap gene *giant* and the





Role of the PAR/CES-2 subfamily of bZIP transcription factors in the transcriptional regulation of programmed cell death (apoptosis). According to this model, PAR/CES-2 proteins compete for transcriptional activation (►) or repression (■) of target genes that can inhibit cells

from undergoing programmed cell death during development. Based on our current understanding, *ces-1* could be either a target gene that is directly or indirectly repressed by CES-2 (as shown here) or another transcription factor whose activity is dominantly repressed by CES-2.

mammalian gene *E4BP4* (refs 7–11).

DBP, TEF/VBP and E4BP4 were originally identified as tissue-specific DNA-binding proteins, and *giant* was discovered because of its role in *Drosophila* segmentation. HLF was found because in cases of childhood acute lymphocytic leukaemia it contributes part of a fusion gene formed as a result of a DNA transfer between chromosomes 17 and 19. This fusion<sup>12,13</sup> between HLF and another transcription factor, E2A, creates a potent activator of transcription that causes cells to become malignant and whose function depends on both the basic DNA-binding and leucine-zipper domains of HLF<sup>14,15</sup>.

Inaba and colleagues<sup>5</sup> investigated the role of E2A–HLF in the transformation of developing lymphocytes into malignant cells by introducing an inactive form of the fusion product into cells carrying the chromosomal translocation. This inactivated E2A–HLF and as a result the malignant cells underwent apoptosis. The suppressing agent had no effect on the growth and survival of leukaemia cells that did not bear this particular translocation, indicating that E2A–HLF may increase the numbers of developing lymphocytes by preventing their suicide. To confirm this hypothesis, Inaba *et al.* expressed E2A–HLF in mouse pro-B lymphocytes and found that it prevented the apoptosis that would normally occur in response to insults like ionizing radiation.

As CES-2 and HLF are both members of the same subfamily of bZIP transcription factors, it is likely that they are key regulators of cell survival. Each shares a high degree of amino-acid sequence similarity with other family members in both the basic DNA-binding domain and the leucine zipper, so they have almost identical DNA-binding specificity. The three transcriptional activators in this family, HLF, DBP and TEF/VBP, show additional homology in the region immediately

amino-terminal to the basic DNA-binding domain — this region is dubbed the PAR (for proline- and acidic-amino-acid-rich) domain and may help to regulate DNA binding and transactivation by PAR-containing proteins<sup>16</sup>. Replacement of the HLF amino terminus, including the PAR domain, with the E2A transactivation domain may serve to activate HLF-dependent transcription by altering the regulation of DNA binding and/or the transactivational properties of the resulting chimaeric E2A–HLF protein. No PAR domain is present in CES-2 or in the transcriptional repressors Giant and E4BP4. Given the genetic evidence that *ces-2* acts as a repressor of *ces-1*, CES-2 could well be a transcriptional repressor too.

Another feature of the transcription factors HLF, DBP and TRF/VBP is their ability to form heterodimers with one another through the leucine-zipper domain. We still do not know whether CES-2 or either of the transcriptional repressors Giant and E4BP4 can form heterodimers with each other or with PAR proteins. Tissue-specific differences in the transactivational properties of these proteins may also result from differences in their patterns of homodimerization as opposed to heterodimerization, alternative exon usage, and post-translational modification<sup>9–11,14–16</sup>. The close similarity in the binding sites for the factors suggests that genes containing PAR/CES-2 binding sites will be targets for the combined transcriptional regulatory effects of this set of transcription factors<sup>4,14–16</sup> (see figure).

Whether the transcriptional targets of the PAR/CES-2 family include genes previously identified as central regulators of apoptosis, or represent new genes involved in establishing the sensitivity of individual cells to apoptosis, remains to be seen. Several genes that are key modulators of apoptosis have been shown to be under tight transcriptional control<sup>2,3</sup>. How

PAR/CES-2 proteins regulate the expression of these genes is not known. The identification of *ces-1*, which appears to be genetically repressed by *ces-2*, may shed light on some of these important issues.

Together, these new results suggest that genes that play a role in establishing the sensitivity of specific cells to apoptotic stimuli are under transcriptional control by an evolutionarily conserved family of transcription factors which can have both positive and negative effects on cell survival during development. On the one hand, loss of CES-2 function can result in abnormal neural development. On the other, expression of HLF as part of a constitutively active transcription factor can contribute to the development of leukaemia. For an organism, such defects may result in a cell fate worse than death. □

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# Woman's meat, a man's poison

Peter Little

MANY followers of research into human genetics might be surprised to discover that the gene underlying a single-gene disorder that is arguably the most common in northern Europe has not yet been isolated. Well, perhaps now it has: in this month's issue of *Nature Genetics*, Feder *et al.*<sup>1</sup> report the identification of mutations that occur in a gene in patients with hereditary haemochromatosis (HH). Unlike many candidate genes investigated for their association with hereditary disease, this work has been carried out without recourse to family analysis.

The symptoms of HH are due to a defect in iron metabolism that results in an accumulation of iron in various tissues of the body, leading to failure of liver function, tumours, diabetes and arthritis<sup>2</sup>. The disease can be easily treated—periodic

blood-letting is sufficient. Women therefore rarely develop symptoms until after menopause.

Particularly remarkable is the incidence of this recessive disease: it is very common, with about 1 in 10 northern Europeans being carriers. HH is sometimes misdiagnosed as alcoholic cirrhosis; it is also underdiagnosed because most Europeans have a relatively low meat consumption and so do not develop clinically apparent iron loads. To a woman, the selective advantage of HH may be large: stores of body iron could remain high in the face of adverse dietary conditions and this may explain the extraordinarily high HH frequency—one man's genetic disease may be another woman's salvation during pregnancy and childbirth!

Unusually for a common defect, there is evidence that a large proportion of HH genes are derived from a single ancestral source. Generally, such observations are consistent with a genetic founder effect followed by rapid spread of the mutation through the population to retain the characteristic polymorphic patterns of the ancestral HH chromosome. However, familial analysis has shown that there are low levels of recombination over the whole HH region, which also contributes to conservation of the ancestral pattern. Unfortunately, this has also meant that it has been difficult to refine gene location by classical family studies.

Feder *et al.*<sup>1</sup> have tried to overcome this limitation by using linkage disequilibrium (LD) and identity-by-descent to refine the gene location (see box), so neither knowledge of family structures nor identification of recombinants is necessary. They were able to identify a region of 250,000 base pairs thought to contain the HH gene. Sequence analysis showed the region contained, *inter alia*, a gene, which they named *HLA-H* (H for haemochromatosis, presumably), related to the major histocompatibility complex (MHC) class I gene, *HLA-A*. They went on to show that 83 per cent of 187 HH patients carried a tyrosine instead of a cysteine residue at position 282 (a Cys282Tyr mutation)—another mutation at a different site was thought to be a simple polymorphism. (This new *HLA-H* gene is not to be confused with the existing *HLA-H* (OMIM ref. 142925; see also ref. 2), which is an expressed pseudogene within the MHC cluster.)

Feder *et al.* argue that the remaining patients, who did not have any mutations in *HLA-H*, acquired the disease by virtue of mutation in a second, unidentified locus; that is, the genetic basis of HH is heterogeneous and a second HH gene must map to another chromosome region.

How likely is it that an *HLA-A*-related gene causes HH? Curiously, transgenic mice that lack  $\beta_2$ -microglobulin<sup>3</sup> develop iron overload similar to HH, and as  $\beta_2$ -microglobulin complexes with MHC class I proteins, this, rather indirectly, suggests a role for *HLA-H* in iron loading.

The data look compelling, but Feder *et al.* state that "formal proof must await functional studies....". Why this caveat? The principal problem is linkage disequilibrium: if this was complete, then a mutation in the gene, say immediately next to the HH gene, would behave exactly like the HH gene in these analyses because it could not be separated from the HH gene by recombination. We do not yet have a clear idea of the population dynamics of random DNA sequences, and perhaps the LD of the region is purely a chance accident of these dynamics. Experimentally, LD is dependent on differences between

## Strategy for tracking down the HH gene

If a mutation has occurred once in a population and then spread through it, as is the case for many genetic diseases, then the analysis of linkage disequilibrium (LD) can be used to refine the disease gene's location on a particular chromosome. Let us say there are a series of ten polymorphisms along the region of chromosome 6 that contains HH: call these A/a to J/j: in all cases, the upper-case form is found in 90% ( $P=0.9$ ) and the lower-case form in 10% ( $P=0.1$ ) of the population (see table below). The actual chromosomal arrangement of polymorphisms (AbCDEfghij, for example) is called the haplotype. Further, assume the HH gene is located between E/e and F/f. The original HH mutation must have occurred on a chromosome with a given haplotype—say all lower-case a-j. If we now analyse the polymorphisms on the HH-bearing chromosome, we

would see a frequency of 1.0 for each lower-case polymorph instead of the expected general-population figure of 0.1: there is a large probability excess over the frequency in the non-HH population. Some recombination would have inevitably occurred in HH chromosomes as they spread through the population: thus, the excess probability of a and j (furthest from HH and therefore most likely to have recombined) might be reduced compared to e and f, which flank the HH gene. In fact, the closer to HH you get, the greater will be the excess. (Good examples of LD mapping and associated mathematics can be found in refs 7 and 8.) Additionally, analysis of individual disease haplotypes can then be used to determine a region which is identical by descent (IBD) even in variant haplotypes and therefore likely to contain the HH gene. P. L.

| Ancestral  | a   | b   | c   | d   | e      | HH | f      | g   | h   | i   | j   |
|------------|-----|-----|-----|-----|--------|----|--------|-----|-----|-----|-----|
| Variant 1  | A   | B   | c   | d   | e      | HH | f      | g   | h   | i   | J   |
| Variant 2  | A   | B   | C   | D   | e      | HH | f      | g   | h   | I   | J   |
| Variant 3  | A   | B   | C   | d   | e      | HH | f      | G   | H   | I   | J   |
| Variant 4  | A   | b   | c   | d   | e      | HH | f      | g   | H   | I   | J   |
| $P_{Obs.}$ | 0.2 | 0.4 | 0.6 | 0.8 | 1.0    |    | 1.0    | 0.8 | 0.6 | 0.4 | 0.2 |
| $P_{Exp.}$ | 0.1 | 0.1 | 0.1 | 0.1 | 0.1    |    | 0.1    | 0.1 | 0.1 | 0.1 | 0.1 |
| IBD        | A/a | B/b | C/c | D/d | e only |    | f only | G/g | H/h | I/i | J/j |

Idealized linkage disequilibrium: alleles are indicated in upper or lower case. Frequency ( $P$ ) of all lower-case alleles in the normal population is 0.1. Five chromosome variants carrying HH are shown; observed and expected  $P$  values for lower-case alleles are listed. The peak linkage disequilibrium is over alleles e and f—this is the only region common to all haplotypes and it shows identity by descent. Red, ancestral HH haplotype; blue, non-ancestral.



disease and control groups: these have to be selected carefully to avoid familial or ethnic biases in polymorphic frequencies. Finally, in the patients without mutations in *HLA-H*, Feder *et al.* invoke genetic heterogeneity as an explanation, though this should always be used with great caution to explain discordant results. Their caveat is therefore realistic and responsible.

There is also evidence against some of their conclusions. Powell *et al.*<sup>4</sup>, and L. W. Powell and E. C. Jazwinska (personal communication) have *HLA*-typed 100 large families containing 1,467 individuals, of which 245 are affected with HH. *HLA* is linked to HH on the short arm of chromosome 6 (designated 6p), and in all individuals the inheritance pattern is concordant with a 6p-linked gene. This does not support heterogeneity and suggests that the second HH locus, if it exists, must also be on chromosome 6p.

Even more curiously, there are at least two cases of recombination that place the HH gene proximal to the *HLA-H* gene by at least 500 kilobases or so, but in these cases the mutation status of *HLA-H* is unknown (ref. 5, and E. C. Jazwinska, personal communication). Does this mean that *HLA-H* is not the HH gene? Certainly, I believe that this is a real possibility, but it is not necessarily the case: the whole region shows high LD. In principle, there could be a gene or genes located within the high LD on 6p and proximal to *HLA-H*. Close linkage of genes that independently contribute to a phenotype has been seen before: for example, genes predisposing to Beckwith-Wiedemann syndrome are clustered on chromosome 11p15 (ref. 6). Maybe HH is a tightly linked polygenic disorder.

Familial analysis may not have identified the *HLA-H* gene, but it can be used to disprove its candidacy — it is only necessary for a recombination to separate the Cys282Tyr mutant and the disease phenotype, and the game is thrown wide open for a second time. The recombinants have been identified — wait for the results! □

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## A fresh glass of frozen chaos

Peter G. Wolynes

A TRIUMPH of modern theoretical physics is the notion that rigidity and orderliness go together<sup>1</sup>. A periodic array of atoms is recognizably and globally different from a fluid; pushing at it in one place causes no rearrangement, because a local disturbance will be met by a collective response of the whole. The rigidity of glass, an amorphous solid, is more mysterious — without any apparent order, a chaotic jumble of atoms behaves as if rigidly

motions convert one jumbled arrangement into another, allowing flow?

Several approaches from the dynamical theory of liquids suggest a route to thermal stability for frozen chaotic configurations<sup>4-8</sup>. Chaotic density patterns may be unstable even to small thermal motions at high temperature, but become locally stable to vibrations, as Bernal's packings were, below a so-called dynamical transition temperature. The pattern is still not

rigid, however — at temperatures below this point the flow mechanism simply changes. Instead of single-particle flow, a small region must be replaced by another, locally stable, aperiodic packing in order for flow to occur. The total number of such packings is large, but the low-energy ones are rare; so, as the temperature is lowered further, the entropy decreases rapidly. As there are then so few possible modes of rearrangement, the changes between structures become more difficult, and the system more rigid.

Eventually, the simple theories predict a real entropy crisis, where even on the longest timescales there is essentially a single aperiodic crystal instead of many alternative chaotic patterns. A true phase transition would occur if the system could experimentally be maintained in equilibrium. In contrast with freezing into a periodic crystal, this 'random first-order transition' has no latent heat, because the frozen state is hardly different from the other competing amorphous packings.

These theories make many approximations and are therefore controversial. Random first-order transitions do occur, however,

in some exactly solvable models of disordered magnets called mean-field Potts spin glasses<sup>9</sup>. In these rather abstract models, the relevant degrees of freedom are vectors assigned to impurity sites at random locations. They each point in one of a finite number of possible directions, and the couplings between the vectors are infinite in range, and favour either parallel or skew orientations, assigned at random. The vectors cannot decide which of the conflicting interactions to satisfy, and that prevents, or 'frustrates', the formation of a globally ordered state.

While the mathematics of these models is clear, the physics is not entirely transparent. Is it the frustration of the interactions alone that causes the random first-order transition, or is the original,



Andy Sacks/Tony Stone

A glass goblet. Its rim may be passing through a subtle phase transition which absorbs no heat, changing from an amorphous solid that can only alter its structure by collective molecular rearrangements, to a true liquid.

frozen. But a paper in the June issue of *Physical Review Letters* by Chandra, Feigelman and Ioffe<sup>2</sup> promises to illuminate this puzzle by showing how the same problem of frozen chaos can be studied in a very different context from amorphous solids: a periodic array of superconducting wires in a magnetic field, coupled through the Josephson effect. A frozen but disordered pattern of superconducting order can arise in this system, by a mechanism thought to be like that in glasses.

Without thermal motion, rigidity and chaos can easily coexist. By compressing ball-bearings in an elastic bag, the crystallographer J. D. Bernal constructed mechanically stable but randomly packed collections of hard spheres<sup>3</sup>. But on the atomic scale, why don't random thermal

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intrinsic disorder of the exactly solved model essential? The infinite range of the interactions is needed for the analysis to be exact, but how is the dynamical transition smeared for the finite-range interactions of structural glasses? Is the underlying equilibrium transition still present for a short-range interacting system? To understand the frozen chaos of an amorphous solid, these issues must be faced.

Chandra *et al.* describe an experimentally realizable model where these issues can be studied in a controlled manner. Its fundamental dynamical objects and interactions seem far from the interacting molecules of an amorphous solid. The system is a stack of two mutually perpendicular sets of parallel superconducting wires, with a tunnel junction at each intersection, and the dynamical variables comparable to the atom positions or density waves of the glass are the quantum-mechanical phases of the superconducting-order parameter in each wire. The interaction energy is the Josephson coupling at the junctions, which tries to equalize the phases on any two intersecting wires.

This attempt to minimize the Josephson energy is frustrated, however, by a magnetic field imposed externally, which by itself would lead to an oscillation of the phase. Like the molecular system, the energy function of this system is frustrated, but it possesses no intrinsic disorder or randomness. On the other hand, like the exactly solved magnetic models, the interactions extend over the size of the complete array, so that an exact mathematical analysis is possible — and this system can actually be built and studied in the laboratory.

The analytical study made by Chandra *et al.* shows that this system has transitions like those in the approximate theories of the liquid-glass transition. There is a crisp dynamical transition at a higher temperature than the first-order random equilibrium transition<sup>10</sup>. At the dynamical transition, many chaotic patterns of superconducting order become locally stable. Both transitions are discontinuous, but there is no symmetry change. So frozen chaos can be realized through a random

first-order transition in a system with no intrinsic disorder in the energy function.

The experimental realizability of the Josephson array system is important. Glassy phenomena occur on long timescales and are therefore difficult to simulate economically by computer, and laboratory experiments usually give only a limited, macroscopic-average view of the dynamics. In contrast, the Josephson array can be interrogated element by element like any solid-state circuitry, so it can be used as an analog computer to study all the other fundamental questions about glassiness. Intrinsic disorder can be easily introduced into the fabrication to study

its effect. Severing some connections between the wires is also possible, so the interactions can be made short range like those in a structural glass.

The Josephson array will be a test-bed for the new notions about how chaotic patterns, fluctuations and rigidity can co-exist. Like the versatile concept of broken symmetry, these ideas about frozen chaos may have implications in areas of physics far from the study of amorphous solids where they arose. □

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## SELF-REPLICATION

# Even peptides do it

Stuart Kauffman

ON page 525 of this issue<sup>1</sup>, David Lee and colleagues describe what appears to be the first case of a self-replicating peptide, a result that may prove to be either a mere chemical curiosity, or seminal.

The authors show that a 32-amino-acid peptide, folded into an  $\alpha$ -helix and having a structure based on a region of the yeast transcription factor GCN4, can autocatalyse its own synthesis by accelerating the amide-bond condensation of 15- and 17-amino-acid fragments in solution (see Fig. 1 on page 525).

The design of this replicator was based on a protein found in nature, an  $\alpha$ -helical coiled coil. Reasoning that a given  $\alpha$ -helical subunit of the entire structure could be seen as a complementary binding surface, acting cooperatively to organize other participating peptide subunits in the coiling, the authors hoped that a similar 'template' function could be found in smaller fragments. The ligation, or joining, site was constructed so as to lie on the solvent-exposed surface of the  $\alpha$ -helical structure of their 32-amino-acid sequence.

Lee *et al.* established autocatalysis by showing that by increasing initial concentrations of the 32-amino-acid template, with constant concentrations of the 15- and 17-amino-acid substrates, a marked increase was produced in the initial rates of template production. The increase correlates with the square root of the initial template concentration, as seen in self-ligating polynucleotide systems<sup>2,3</sup>. The reaction is region- and chemically selective, yielding less than 15% side products, and proceeds through the major autocatalytic pathway open to the system.

Do these results reflect a rare chemical quirk in the repertoire of peptides and polypeptides, or might they hint at a route to self-reproducing molecular systems on a basis far wider than Watson-Crick base-pairing in polynucleotides? At this stage,

we cannot know, but the way is now open to investigate.

The first step, beyond independent verification of the reaction system, is to construct self-reproducing cross-catalytic systems of two peptides, A and B, where A catalyses the formation of B from B's two fragment substrates, and B does the same for A. Such a system would be collectively autocatalytic — no molecule would catalyse its own formation, but the system would collectively catalyse its own formation from 'food' sources — here, the two A fragments and the two B fragments. If collectively autocatalytic peptide sets with two catalytic components can be constructed, can systems with three, four or hundreds of cross-coupled catalysts be created?

Such experiments are important. A free-living cell, prokaryote or eukaryote, is in fact a collectively autocatalytic system — virtually no molecule, including DNA, catalyses its own formation. Most of the cell's catalysts are proteins, so if collective autocatalysis in complex peptide systems is possible, we would have a new model for self-reproducing systems.

A host of experimental and theoretical questions about such systems present themselves. If such systems can be created, is it possible in general to constrain the side reactions enough for the autocatalytic system to increase in concentration as the fragment 'fuel' is added? Would such an increase be aided by confining reactions to a surface, or within a small volume? Are there means other than thioester promotion to drive the synthesis of peptide bonds? Even if such systems can be designed using clever synthetic chemistry, is the spontaneous formation of collectively autocatalytic sets of peptides rare or common as a function of the diversity of the peptides, and of the regions of sequence space they are derived from? Can such autocatalytic systems be

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constructed or spontaneously assembled from mixed polymer systems consisting of DNA, RNA, peptides and perhaps other polymers and their building blocks? Can such systems evolve to 'neighbouring' autocatalytic systems while retaining 'catalytic closure', and could current life have evolved from one?

The new autocatalytic ligation-reaction system is merely exergonic: left to its own devices, the system will simply run to equilibrium. Can an autocatalytic system be created that carries out thermodynamic work cycles whereby the system sustains displacement from equilibrium, performs coordinated work and achieves such coordination by controlling, constraining and 'correcting' unwanted side reactions (as in DNA editing and repair; P. W. Anderson, personal communication, and ref. 4) to enhance its own rate of reproduction?

The dominant view of life assumes that self-replication must be based on something akin to Watson-Crick base pairing. The 'RNA world' model of the origins of life conforms to this view. But years of careful effort to find an enzyme-free polynucleotide system able to undergo replication cycles by sequentially and correctly adding the proper nucleotide to the newly synthesized strand have not yet succeeded<sup>5,6</sup>.

A polynucleotide system based on a ribozyme polymerase able sequentially to add the correct nucleotides (and thus copy itself) might work. In contrast, the simple and successful reproducing molecular systems described by Lee *et al.*<sup>1</sup> and by von Kiedrowski<sup>2</sup> which uses a single-stranded DNA hexamer and its two trimer fragments) are based on a polymer catalysing its own formation from two fragments. Both show that autocatalytic systems based on specific ligation reactions are possible. Because a variety of polymers and small molecules can catalyse such reactions, these results may prove seminal: the creation or spontaneous formation of simple or collectively autocatalytic sets may occur far more readily than we thought. Given the emerging field of 'molecular diversity', with its capacity to synthesize high-diversity DNA, RNA and peptide libraries<sup>7</sup>, these questions are now open to detailed scrutiny. □

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## Attention is everywhere

Kenneth H. Britten

VISUAL attention is one mechanism that enables us to emphasize important objects or spatial locations over less important ones. This process has been the target of extensive scrutiny owing to its strong appeal as an example of 'higher' cognitive processing that is fairly straightforward to define, manipulate and measure. But despite the intensity of our attention, our understanding of its physiology remains patchy. Until now, for example, we have known next to nothing about the effects of attention on the pathway to the parietal lobe, even though this appears to be one of the key loci controlling spatial attention<sup>1</sup>.

This lacuna is now well on the way to being filled by the work of Treue and Maunsell, which appears on page 539 of this issue<sup>2</sup>. In a simple and elegant experiment using alert monkeys, they carefully

measure the effects of directed attention in two structures on the pathway that leads to the parietal lobe. By recording from motion-sensitive neurons while the monkey attends to one of two or three simultaneously moving dots, these authors discovered that impressive modulatory processes are in operation which are as large as any seen elsewhere in the visual system. In their experiments, the attended stimulus takes greater control of cells' responses than does the unattended stimulus; they are about equal in the absence of directed attention. Such an effect has been seen previously in other visual areas, and raises the simplifying prospect that one process or set of mechanisms supports selective attention throughout the visual system.

The primate visual system is organized into two largely separate pathways, ►

## An unhealthy volcanic spring



THE destructive armoury of volcanoes includes landslides, rivers of lava and glowing clouds of pumice, ash and hot gas. Floods of water make a strange addition to these, but a paper published last month (C. F. Waythomas *et al.* *Geol. Soc. Am. Bull.* **108**, 861-871; 1996) describes how, some time between 3,400 and 500 years ago, a volcanic crater lake in Alaska burst through its rock walls and emptied its billions of tonnes of water onto the plain below.

The Aniakchak River now flows from that lake's remnant, the well-named Surprise Lake (on the left in the 10-km caldera pictured above), and out of the v-shaped notch in the caldera rim called The Gates. That notch, and the wave-cut terraces inside the caldera showing how large the lake once was, helped lead R. G. McGimsey *et al.*

(*USGS Bull.* **207**, 59-71; 1994) to first postulate a catastrophic flood.

Waythomas *et al.* now show how rapidly it must have happened. To carry the biggest of the boulders found downstream, more than 20 m across, the flow rate must have been more than 100,000 m<sup>3</sup> s<sup>-1</sup>. Analysis of downstream erosion and a dam-break model broadly agree.

Was this a freak event? Other empty calderas around the world have similar rim notches and fan-shaped patterns of deposition downstream. If they too have produced catastrophic floods, then many crater lakes, such as the huge, 40-km<sup>3</sup> Atitlán in Guatemala, may be able to do the same. And at Ruapehu volcano in New Zealand, near a small notch through which a lake drains, the caldera rim is moving outwards by 10-15 mm yr<sup>-1</sup> as the glacier that supports it retreats. S.B.

sometimes called the 'what' and 'where' pathways. These are distinguishable on the basis of anatomical connections, physiology, and the behavioural consequences of restricted lesions. The dorsal 'where' pathway conveys information to the parietal cortex, whereas the more ventral 'what' pathway leads towards areas in the temporal lobe that are thought to be involved in object recognition and visual working memory. Each pathway is organized hierarchically, and Treue and Maunsell explore two areas on the dorsal pathway, near the middle of the hierarchy. These two areas, the middle temporal area (MT, or V5) and the medial superior temporal area (MST), are characterized by a preponderance of directionally selective cells.

The linkage between the analysis of visual motion (revealed by physiology experiments) and the spatial role of the dorsal pathway (neuropsychology) has not been well fleshed out by experiment, but it depends on the importance of visual motion as a cue for image segmentation and determining the depth structure of a scene. Such computations are usually considered low-level, or 'preattentive'. Thus, we can formulate a simple rule: attentional modulation is strong at all levels of the ventral pathway<sup>3-6</sup>, but only at higher levels of the dorsal pathway, after the early, machine-like calculations of object motion. Like many ideas formed in the absence of critical data, this one makes sense, but it is now seriously challenged by the new results of Treue and Maunsell.

These authors have found robust attentional effects as early in the dorsal pathway as area MT, which receives direct input from primary visual cortex, where effects of attention are weak at best. Their critical experiment was very similar in design to the experiments that have shown positive attentional effects in ventral pathway areas. In this design, two dots are moving back and forth within the receptive field of a single neuron. One of these is always moving in the neuron's preferred direction and the other in the opposite direction. Thus, the purely visual input to the cell is approximately constant, and indeed if the monkey's attention is elsewhere, the response of the neuron is constant too. However, if the monkey is cued to attend to one object and detect a small speed change, then neurons in both MT and MST show modulated discharge, increasing their firing when the attended object is moving in the preferred direction, and reducing it when the object reverses direction (despite the fact that the other object is now moving in the preferred direction). The amplitude of the modulation provides a measure of the strength of the attentional signals, and these are strong. In MT the firing rates changed by about a factor of

two, in MST slightly more. The somewhat larger effects in MST come as no surprise because of its higher location on the cortical hierarchy, but what is surprising is the magnitude of the effects in MT. The magnitudes in both areas are roughly equal to those seen on the ventral pathway, in V4 and inferotemporal cortex.

Now that similar measurements have been made in both pathways, with similar results, we can conclude that attentional signals have approximately equal roles in both, and that there is nothing uniquely privileged about motion analysis or the dorsal pathway. This raises a question: if MT carries out preattentive operations, how do these work if the signals there are attentively modulated? Psychophysical evidence suggests that attention can influence very low-level aspects of motion processing, such as the motion after-effect<sup>7</sup>, so the concept of invariant preattentive operations might itself be questionable.

Also, how would tasks that depend on integration across multiple objects remain unaffected? Other results<sup>8</sup> suggest that at least one complex motion task — the recovery of self-motion from optic flow — is unaffected by directed attention to single objects. This seems puzzling if the distributed signals on which this task depends are being altered by selective attention to a small part of the image. The heterogeneity of single-cell attention effects might provide some answer to these questions: some cells are only minimally modulated, and these might serve to support operations whose output is invariant with attention.

Pondering the mechanism that underlies the results of this and most other studies, one notes that attentional effects are most evident within a single receptive field. This suggests the existence of some form of control or selection mechanism operating fairly early in the cortex<sup>9</sup>. The attentional control signals in most studies are driven by 'top-down' (or endogenous) cues, and how these signals reach back into early stages of processing to select certain synaptic inputs over others remains a profound mystery. □

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DAEDALUS

## Boll-weevils

THE world's most abundant polymer is probably not cellulose but chitin, the structural material of insects and crustacea. Chitin is merely cellulose with an acetamino group on each glucose moiety; it is probably tougher and more durable than cellulose itself. However, unlike cellulose, it doesn't come in conveniently fibrous forms. So while we exploit cellulose heavily in timber, paper, textiles and many other products, we ignore chitin. Daedalus is now remedying the omission.

He recalls that rayon, and the new fibre Tencel, are made by dissolving natural cellulose in a suitable solvent, and squirting the liquid into fibres. So DREADCO chemists are treating crab shells and insect wing-cases with cuprammonium hydroxide, alkaline xanthate, morpholine *N*-oxide and other promising solvents, hoping to dissolve chitin also. They will then squirt the solution into an evaporator or a precipitating reagent, giving chitin fibre.

Chitin fibre will be much springier and tougher than cellulose, better suited for sacks and tyre cord than *haute couture*. The obvious source of raw material is some sort of crab farm or beetle ranch. The resulting trickle of chitin would be far too expensive for bulk processing. It might best be exploited as an additive for the cheaper cellulose. A dash of chitin in the rayon or Tencel solution should toughen the final fibre greatly without raising its price too much.

DREADCO's chemists, however, plan to make chitin in bulk. Cellulose is easy to acetylate. They are treating it with reagents such as sodamide and ketene, hoping to *N*-acetylate it to chitin. Ideally, they would like to convert only a limited fraction of the glucose units in the cellulose chain. The result would then be a true copolymer of cellulose and chitin, with the virtues of both materials.

This new chemistry might be ideal for 'case-hardening' wood. One wipe with the DREADCO mixture, and your wooden table would acquire a splendid, durable 'chitinized laminate' surface. But Daedalus's main goal is an artificial leather. Like chitin, leather is a two-dimensional skin product: tough and durable, crack-proof, resistant to abrasion. A soft wood-pulp fibre-board could be formed into boots, jackets, upholstery, or whatever, and then cross-linked and toughened by chitinization into uniform, seamless, artificial-leather products. Furthermore, their copolymeric molecules would never rot. Some microorganisms have the enzymes to attack the chitin of dead insects, and many can degrade cellulose. But none can do both at once.

David Jones



# Frameshift mutator mutations

**SIR**—Human tumour cells of the microsatellite mutator phenotype (MMP) accumulate many thousands of somatic mutations in simple, repeated nucleotide sequences<sup>1</sup>. The MMP is a landmark of hereditary non-polyposis colorectal cancer (HNPCC) and a significant fraction of other sporadic tumours of the gastrointestinal and urogenital tracts<sup>2</sup>. MMP tumours exhibit a low frequency of mutations in the p53 tumour-suppressor gene<sup>1</sup>. In contrast, inactivation of the type II transforming growth factor- $\beta$  receptor in gastrointestinal cancer of the MMP commonly occurs by frameshifts in a poly(A) nucleotide track in the gene's coding region<sup>3</sup>. Human tumours of the MMP have been associated with defects in the human homologues (*hMSH2* and *hMLH1*) of the *Escherichia coli* *MutS* and *MutL* families of DNA mismatch-repair genes, respectively<sup>2</sup>; *hMSH3* (also called *DUG*) and *hMSH6* (also called *GTBP*) are two other *MutS* genes<sup>4,5</sup>. The functions of DNA mismatch recognition and repair enzymatic complexes in humans are poorly understood. In the yeast *Saccharomyces cerevisiae*, MSH3 and MSH6 are part of the MSH2-dependent repair pathway, contributing to the stabilization of microsatellite sequences<sup>6,7</sup>. We have found that tracks of 8 deoxyadenosines and 8 deoxycytosines present in the coding regions of *hMSH3* (codons 381–383) and *hMSH6* (codons 1,116–1,118), respectively, are hotspots for frameshift mutations in MMP tumours.

We used DNA isolated from 41 colorectal tumours of the MMP to amplify by PCR (polymerase chain reaction) a region encompassing the (A)<sub>8</sub> and (C)<sub>8</sub> tracks of *hMSH3* and *hMSH6*, respectively. We found PCR bands with altered mobility in 39% (16/41) and 30% (12/40) of these tumours for *hMSH3* and *hMSH6*, respectively (see figure). Sequencing analysis confirms that frameshifts of one nucleotide in these monotonic runs in *hMSH3* (all deletions) and *hMSH6* (6 insertions and 6 deletions) accounted for the band shifts.

These frameshift mutations are not restricted to colon tumours. We found *hMSH3* mutations in stomach (3/5) and

pancreas (1/2) adenocarcinomas of the MMP and in 1 of 2 uterine and 1 of 18 prostate carcinomas. We identified *hMSH6* mutations in two gastric tumours of the MMP. In contrast, we found no frameshift mutations in *hMSH3* or *hMSH6* in 70 gastrointestinal tumours without enhanced

Frameshift mutations in the *hMSH3* and *hMSH6* genes in colon tumours and tumour cell lines. Regions encompassing the (A)<sub>8</sub> and (C)<sub>8</sub> tracks in the *hMSH3* (a) and *hMSH6* (b,c) genes were amplified by PCR using DNA isolated from normal (left) and tumour (right) tissue from the colorectal cancer cases and tumour cell lines indicated at the top. Tumour cell lines of the MMP (colon except when indicated) are: DU145 (prostate) (A); LS411 (B); LS174-T (C) in a; and LoVo (1), DU145 (2), LS411N (3), SKUT-1B (uterus) (4), HCT116 (5), SW48 (6), Cal51 (breast) (7), LS180 (8), DLD-1 (9), AN3CA (endometrium) (10), SK-OV-3 (ovary) (11) and HCT15 (12) in c. The origins of these tumour cell lines has been previously described<sup>9–14</sup>.

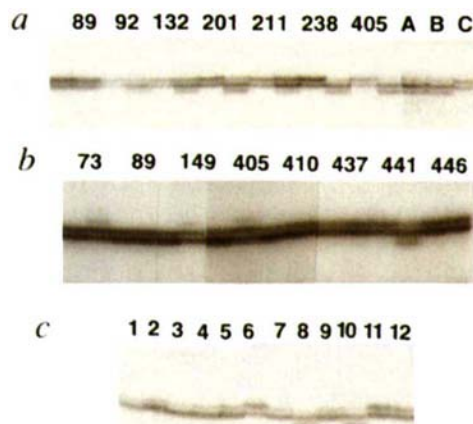
PCR primers were 5'-AGATGTGAATCCCCTAATCAAGC-3' and 5'-ACTCCCACAATGCCAATAAAAT-3' for *hMSH3*, and 5'-GGGTGATGGTCTATGTGC-3' and 5'-CGTAATGCAAGGATGGCGT-3' for *hMSH6*. Cases 92, 132, 201, 211, 238 and 405, and cell lines A and B, are positive for frameshift mutations in the *hMSH3* gene (a), and cases 73, 149, 405, and 441 and cell lines 6, 8 and 10 are positive for mutations in the *hMSH6* gene (b, c). Tumour 92 also contains a somatic G-to-T transversion at codon 580 of the *hMSH2* gene that changes an Asn to a stop codon (GAA>TAA). PCR products appear as a double band because of the variable incorporation by *Tag* polymerase of an extra nucleotide (most frequently deoxyadenine) at the end of the synthesized DNA fragment<sup>16</sup>. This makes the determination of the heterozygous or homozygous nature of the mutations more difficult.

microsatellite instability (0/70 versus 16/25 for *hMSH3* and versus 12/28 for *hMSH6*, in both cases  $P < 0.0001$ , Fisher exact test).

The frameshift mutations in the *hMSH3* (A)<sub>8</sub> and *hMSH6* (C)<sub>8</sub> tracks appear to be selected for during tumorigenesis because they are absent in other similar repeated sequences, including A<sub>8</sub> tracks, in the coding regions of the *hPMS2* mismatch-repair and the DNA polymerase  $\alpha$  genes, and in an intron of the retinoblastoma gene and of the fibronectin type III gene ( $P < 0.001$ , Fisher exact test), and (G)<sub>8</sub> tracks in the 3'-end untranslated exon of the nerve growth factor receptor gene. We detected two mutations in a (G)<sub>8</sub> track in the promoter region of the muscle glycogen synthase gene (2/39 versus 12/28,  $P = 0.002$ ,

Fisher exact test). Therefore, *hMSH3* and *hMSH6* frameshift mutations are frequent events specifically associated with cancer of the MMP.

Human *MSH3* mutations are also present in LS411N and DU145 tumour cell lines, and *hMSH6* mutations in AN<sub>3</sub>CA, SW48 and LS180 and LS174-T cell lines (see figure, and data not shown). Mutations in *hMSH6* are the first mismatch-



repair defects to be associated with the LS180 and LS174-T cell lines (both derived from the same tumour<sup>9</sup>) which show frequent slippage-induced<sup>8</sup> insertions/deletions at microsatellites<sup>9,10</sup>. Mutations in the *hMSH6* gene have been reported in the DLD-1 cell line, which has infrequent dinucleotide microsatellite mutations<sup>9,11</sup> but a high incidence of single-base substitutions<sup>12,13</sup>. Cell hybrids between HCT-15 (probably isolated from the same tumour as DLD-1; ref. 9) and LS174-T regain stability at dinucleotide microsatellites<sup>14</sup>. From these observations we conclude that there are still unknown factors inside or outside the DNA mismatch-repair gene family that influence microsatellite replication fidelity.

The SW48, AN<sub>3</sub>CA and DU145 tumour cell lines have been reported to carry defects in the *MutL* gene family<sup>13</sup>. Therefore, our results show that these tumour cells contain inactivating mutations in at least two different DNA mismatch-repair genes. Tumours containing two mutated genes are also relatively common, including one *hMSH2/hMSH3* and eight *hMSH3/hMSH6* double mutants (for example, tumour 405 in the figure). ►

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The origin of the frameshift mutations in the *hMSH3* and *hMSH6* genes is not clear. They may be the result of a previous defect in mismatch repair because they occur at hotspots for slippage-generated mutations<sup>8</sup>. If this hypothesis is correct, it would imply that the MMP unfolds in consecutive steps. Rare homozygous mutations in a DNA mismatch-repair gene (such as *hMSH2* or *hMLH1*) may lead to mutations in other mismatch-repair genes as they contain targets for the mutagenic action of the first mutator mutations<sup>1</sup>. Because these slippage-induced mutations are much less frequent in other genes containing (A)<sub>8</sub> and (G)<sub>8</sub> tracks, the frameshift mutations in these secondary mutator genes appear to be under a positive selective pressure during tumorigenesis. These mutations may contribute to the genomic instability of the

tumour cells. This enhanced (or broader) genomic instability may accelerate even more the accumulation of mutations in cancer genes during tumour progression.

This progressive model of mutator mutations helps to explain the redundancy in DNA mismatch repair and its causal relationship with cancer of the MMP, and the temporal relationships and hierarchies among different mutator genes<sup>2,6</sup>. This model may also help account for the phenotypic properties of tumour cells of the MMP<sup>1-3,13</sup> and for the rarity of occurrence of tumours in homozygous knock-out mice for mismatch-repair genes<sup>15</sup>.

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and measured the gamma dose rate *in situ*. We used a large sediment sample from a hippopotamus mandible for the calculation of the gamma dose rate of the spring collection samples. We checked OSL bulk samples for radioactive disequilibrium using high resolution gamma spectrometry.

The samples from the third test pit, which were usually small tooth fragments, show considerable spread of ESR results within layers (*a* in the figure), indicating re-working of material by spring action. The age spread is smallest for the Middle Stone Age human occupation horizon (121,000±6,000 years) where the taphonomy clearly indicates a short-term event, and re-working can be excluded. The samples of the spring collection (*b* in the figure) yielded age estimates from about 100,000 to 300,000 years old. Because the precise locations of the specimens were not recorded, the age scatter can be explained by the continuous spring activity.

To tighten the age of the Florisbad hominid, we directly analysed the associated hominid tooth. It is the only dental sample clearly associated with the other hominid remains, and its stratigraphical position has been recorded<sup>1</sup>. A newly developed ESR technique allows the measurement of dental fragments without any further destruction<sup>7</sup>. We separated and analysed two fragments (10.5 and 25 mg), subsequently re-inserting them into the original specimen. For age calculation, we used saturation water contents, a cosmic dose rate for a depth of 5±1 m and the average concentration of radioactive elements of white sand layers. All other teeth of the spring collection had low uranium concentrations (enamel: 3–101 p.p.b. (parts per billion); dentine: 10–268 p.p.b.), so we assumed the human tooth to be within these limits. Considering all uncertainties, we obtained a result of 259,000±35,000 years old (weighted mean).

The ESR and OSL results (*c* in the fig-

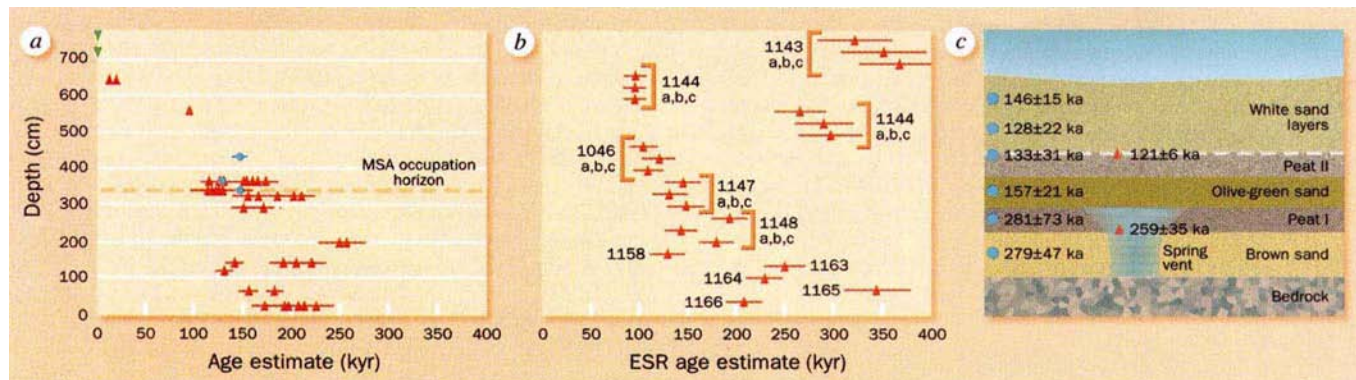
## Direct dating of Florisbad hominid

**SIR** — In 1932, hominid remains were found at the Florisbad spring, near Bloemfontein, South Africa<sup>1</sup>. The partial cranium which shows damage by hyena chewing was part of a natural accumulation of mostly carnivore prey remains, which were trapped in vertical spring vents. The spring and associated white-sand bodies intruded horizontal sand and clay layers when the spring was active in a specific locality. When the spring migrated to another area, these vents were sealed by the horizontal deposits, resulting in a succession of ancient spring vents at various depths (and age). The horizontal deposits are interbedded with dark organic clay horizons (peats). "Peat II" represents an old land surface on which Middle Stone Age artefacts and bones occur<sup>2</sup>.

The hominid remains consist of much of the frontal bone and right side of the face, together with parts of the parietals, maxillae and the right M<sup>3</sup> tooth. Based on

the last reconstruction in 1983 (ref. 3), the Florisbad fossil is most reasonably classified with other late archaic specimens such as Ngeloba, Eliye Springs, Omo 2 and Singa from East Africa and Jebel Irhoud 1 and 2 from North Africa. Most of these fossils have age estimates in the range of 100,000 to 200,000 years old<sup>4</sup>.

Previous dating attempts produced infinite radiocarbon results for the human occupation layer and a uranium-series estimate of >100,000 years for "Peat I" (ref. 3). Our first ESR (electron spin resonance) study followed conventional procedures<sup>5</sup>, which involves grinding of large portions of tooth enamel (200–400 mg). We collected one set of tooth samples *in situ* from the third test pit which spans the whole sedimentary sequence at Florisbad, and a second set from the original spring collection<sup>1,2</sup>. We carried out optically stimulated luminescence (OSL) dating<sup>6</sup> on quartz separates from sediment samples



*a*, Age estimates for the third test pit (the results of the occupation horizon are projected into the sequence) (ages are in thousand years) *b*, Tentative ESR age estimates of the spring collection samples. *c*, Schematic diagram of the lower part of the sediment sequence at Florisbad with a reconstruction of the spring vent yielding the hominid fossils (not to scale). The spring mount dissected the basal sand and Peat I and was covered by the olive green sand. The ESR results yield an age of 259,000±35,000 years for the human tooth and an age of 121,000±6,000 years for the younger human occupation horizon on top of Peat II. Although having larger errors (because of saturation problems), the OSL age estimates confirm the large age span between the beginning of sedimentation to the deposition of the occupation horizon. Green triangles, radiocarbon; red triangles, EU-ESR; blue circles, OSL.

ure) support an age for the occupation horizon corresponding to the last interglacial (125,000 years ago). The hominid is clearly older, perhaps coinciding with the previous interglacial. Our results confirm the view that Peat I and Peat II were separated by an entire landscape cycle and that the hominid remains may relate to an earlier Middle Stone Age industry<sup>8</sup>.

The relatively old age of the hominid fits well with its combination of archaic and modern characteristics, considering that anatomically modern humans are known from several southern African sites from the last interglacial<sup>4</sup>. We are confi-

dent that this new method of direct ESR analysis will allow age estimates for further enigmatic hominid fossils such as those from Broken Hill, Skhul and Tabun.

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body image, for example, they could name their own as well as other people's body parts (no autotopagnosia) and they could perform skilled movements (for example, touching their nose; waving goodbye; pretending to stir sugar in a cup of tea) with their right hands in response to commands (no apraxia). Nor did they have left parietal or frontal lesions of the kind that might be expected to produce such disturbances. The failures cannot therefore be attributed to simple body-part confusion or to general confusion about limb movements.

We conclude that at least some anosognosic patients will refuse to acknowledge the paralysis of another patient<sup>4</sup>. The observation raises the interesting question of whether the patient needs to believe that the other individual is also a patient. Or, is it the case that some of these patients are unable to access their own body schemata and that such access is necessary even for making judgments about the movements of another human being? Additional experiments are needed to resolve these questions. Certain cells in the monkey frontal lobes<sup>5</sup> respond not only to their own hand performing certain actions but also to the visual image of another monkey's hand performing the same action: would these cells provide the neural substrate for the delusions experienced by these patients?

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## Denial of disabilities in anosognosia

**SIR** — A certain proportion of right-hemisphere stroke patients who have left-sided hemiplegia will vehemently deny their paralysis, even though they may be mentally quite lucid in other respects ("denial" or anosognosia<sup>1-4</sup>). We studied three such patients and found, surprisingly, that two of them also refused to acknowledge the paralysis of a fourth patient (or a "stooge") who was in a wheelchair next to them. Thus, in at least some instances, a patient's denial may generalize to include the disabilities of other people's body movements.

All three patients (L.H., F.D. and L.R.; ages 77, 77 and 78) were right-handed women with a left hemiplegia. Computed tomography scans confirmed the presence of a subacute right middle cerebral artery and right cerebellar artery infarct in F.D.; a right middle cerebral artery and left cerebellar artery infarct in L.H.; and a right frontoparietal infarct in L.R. At the time of testing, L.R. and F.A. were completely lucid mentally (for example, able to orient in time and place; subtract serial twos; digit span; and so on), fluent in conversation, and of average intelligence. L.H. was also fluent in conversation but her digit span was four. Each patient was asked the following sequence of questions repeatedly: can you walk; can you use both hands; can you use your right hand; can you use your left hand; are both hands equally strong? They were deemed anosognosic only if they answered all questions in the affirmative<sup>4</sup>.

Would an anosognosic patient deny another patients' paralysis? Before each experiment, we first verified the patient was mentally alert and was still in denial

as revealed by affirmative answers to the sequence of questions cited above. We then conducted an abbreviated neurological examination on a left-hemiplegic patient in the adjacent wheelchair. (In one case we had to use a student "stooge" pretending to have left hemiplegia.) The wheelchair was in the non-neglected (right) side of the anosognosia patient and was rotated so that the stooge always sat face-to-face with the patient. The patient was then asked carefully to watch the stooge in the wheelchair. From this position, our patient could clearly observe that the stooge's right hand was functioning well but that his left hand was not responding to the examiner's commands.

Immediately after demonstrating the paralysis of the stooges' left hand, each patient was asked "Is that patient moving his arm properly or is he paralysed?" This was done three times in a row and interspersed with brief, irrelevant, distracting questions such as "What do you think of the O. J. trial?" The same experiment was then repeated the following day (L.H. & F.D.) or a week later (L.R.). On all six trials, both L. H. and F. D. responded without hesitation that the other patient was "OK" and that "he is moving his arm up and down". L.R. seemed very surprised by the question, answering "of course he is paralysed; he is not moving his arm", even though she vehemently denied her own paralysis. It is noteworthy, also, that even when L.R. watched her failure to move her arm in a mirror she continued to insist that she was not paralysed.

Finally, we verified that the patients had no problem with other aspects of their

## Biological activity of interleukin-16

**SIR** — Bazan and Schall<sup>1</sup> implied that the predicted existence of an interleukin-16 (IL-16) precursor protein was evidence that the cloned complementary DNA<sup>2</sup> does not represent the originally described native biological activity<sup>3</sup>. But these implications are not supported by our published experimental evidence.

IL-16, formerly known as lymphocyte chemoattractant factor, was identified as a secreted T-cell product that induces motility and interleukin-2-receptor expression in CD4+ T cells; functions that are selectively inhibited by Fab of the OKT4 antibody<sup>4</sup>. IL-16 appears in culture supernatants as a relative molecular mass ( $M_r$ ) ~56,000 biologically active non-covalently linked tetramer, but migrates in monomeric form



in SDS PAGE. Eluted monomeric peptides are inactive but reaggregate to  $M_r$  56,000, regaining biological activity<sup>5</sup>. The protein expressed from the IL-16 cDNA demonstrates all the functions and chemical features of the native protein, including an identical pI, and autoaggregation into functional tetramers. Antibodies against recombinant IL-16 identify native protein of similar size in western blots and block its biological activity. This unique constellation of activities and chemical characteristics is not likely to be fortuitously shared by unrelated proteins.

Further, deletion analysis of recombinant IL-16 demonstrates that all its biological activity resides in the C-terminal 114 residues which are completely within the 130-residue published sequence<sup>2</sup>. Based on sequencing of a murine IL-16 clone, we do agree that IL-16 is synthesized as a precursor molecule, but we cannot confirm the  $M_r$  42,000 size predicted by Bazan and Schall<sup>1</sup> by western blot analysis.

Bazan and Schall argued also that the

presence of GLGF sequences in IL-16 is incompatible with its role as a secreted protein, so is evidence against a specific interaction between IL-16 and its receptor CD4. The function of the GLGF motifs in IL-16 is unclear: however their presence does not provide evidence against secretion of the C-terminal peptide as this motif is found in other secreted proteins<sup>6</sup>. Further, the experimental evidence for a ligand receptor-like relationship between recombinant IL-16 and CD4 is substantial, even if the GLGF sequence is involved in binding.

The structure of IL-16 and its relationship to CD4 raises interesting questions about its role as an interleukin. Its gene structure and chromosomal location are distinct from other interleukins, suggesting that it may indeed have roles other than in the immune system. The binding of IL-16 to CD4 may indicate a role for IL-16 in suppression of HIV-1 replication. Nevertheless, we believe that it is unlikely that IL-16 inhibits HIV-1 replication by competing for binding to CD4, but it may induce CD4-dependent signal-transduction events mediating repression of HIV-1 promoter activity.

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## Tobacco-plant desiccation tolerance

SIR — Holmström *et al.*<sup>1</sup> reported the transformation of tobacco plants with a gene, *TPS1*, which enables the transgenic plants to synthesize trehalose, a disaccharide whose biophysical properties and presence in anhydrobiotic organisms suggests that trehalose produces desiccation tolerance in organisms<sup>2</sup>.

Holmström *et al.* clearly demonstrated an increase in drought tolerance<sup>3</sup> in the *TPS1*-transgenic plants in that the development of drought stress in the protoplast was retarded in the transgenic lines. But their conclusion that, because trehalose concentrations in the cytosol seem too small for osmotic adjustment, stabilization of cellular enhanced structures and macromolecules by trehalose may underlie both the improved water retention and desiccation tolerance, do not seem to me to be completely justified by their data.

There is an explanation other than osmoregulation for the improved retention of water. In their figure (a), Holmström *et al.* showed that drying tobacco leaves display the typical water-loss pattern of detached well-hydrated leaves, that is an initial rapid time-rate of water loss (when stomata are wide open) and a late slow rate of water loss (as drought stress causes stomata to close, thereby restricting

water diffusion from the leaf). Viewed in this way, their figure indicates that, relative to nontransgenic plants, transgenic plants passed into the slow water-loss phase at higher fresh weights, and thus that transgenic stomata commenced closing at milder drought stress; as a result water was retained for a longer time. During plant cultivation then, carbon dioxide supply for photosynthesis would be restricted more frequently by stomatal closure, causing slower growth in the transgenic plants, as noted by Holmström *et al.*

Further, Holmström *et al.* did not report the determination of protoplasmic

drought tolerance of the tobacco lines, therefore no conclusion can yet be reached on their desiccation tolerance. The line 8 transgenic seedlings recovered from 7 hours of drying in air of 50% relative humidity (RH) (followed by 48 hours' rehydration), whereas the control plants failed to recover (figure b of ref. 1). However, Holmström *et al.* gave no measure of the water status of the seedlings at the end of the drying period. The best estimates for seedling water status that can be derived from their data are those of detached leaves at 6 hours' drying (25% RH) (figure a of ref. 1) — line 8 transgenic leaf fresh weight = about 45% of initial fresh weight and control leaf fresh weight = about 32% of initial fresh weight. The protoplasmic drought tolerance of transgenic seedlings then would correspond to some leaf fresh weight less than 45% initial fresh weight and that of nontransgenic seedlings to a leaf fresh weight greater than 32% initial fresh weight. The overlap of these value-ranges permits no conclusion on the relative protoplasmic drought tolerance of the lines. Furthermore, comparison of these drought tolerances in terms of leaf water potential would be necessary, as this parameter is basic to an understanding of plants' water status in relation to air-dryness. A value below –100 MPa is needed for a plant to qualify as desiccation tolerant; the water contents reported above (30–45% fresh weight) would correspond to water potentials of the order of only –10 MPa.

Holmström *et al.*, in producing stable transgenic tobacco plants capable of synthesizing trehalose, report an exciting result. It is to be hoped that with further experimentation on the transgenic tobacco lines they will establish if or how much trehalose contributes to plant desiccation tolerance.

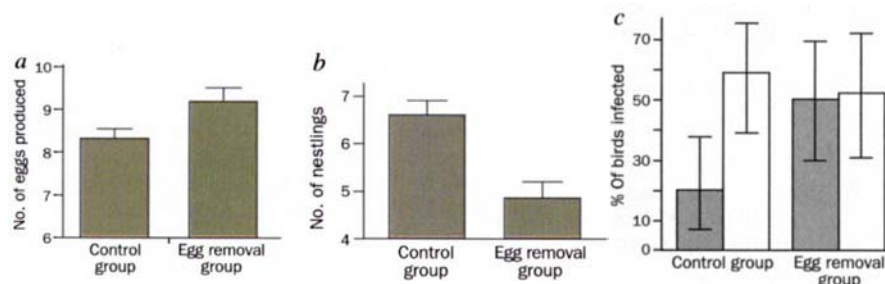
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## Erratum

In the Scientific Correspondence "Clutch size and malaria resistance" by A. Oppliger, P. Christe & H. Richner (*Nature* **381**, 565; 1996), the figure was incorrect. The correct version is shown below.



# Memory's fragile power

Larry R. Squire

**Searching for Memory: The Brain, the Mind, and the Past.** By Daniel L. Schacter. BasicBooks: 1996. Pp. 398. \$27.

THE biology of memory entails two different levels of analysis. The first concerns the cellular and molecular events within neurons that underlie neural plasticity: alterations in neurotransmitter economics at synapses, changes in receptor function, long-term changes in gene expression, and morphological growth and change of dendrites and terminal axonal branching. The second concerns the organization and operation of the brain circuits and brain systems that encode, store and retrieve information. This broad approach, from molecules to systems, would appear at first to describe a full programme of research into the large problem of how the brain learns and remembers. What is missing, of course, from this prescription for research is behavioural work. What is sometimes overlooked, once one is caught up in the excitement and success of neurobiological studies of memory, is the psychological science that defines and describes the richness and the complexity of the phenomenon under study.

Daniel Schacter has written a book about this missing piece. It is a story about the cognitive and practical workings of human memory in the laboratory and in everyday life. An important theme is struck by contrasting memory's importance, its pervasive influence, with its vulnerability to inaccuracy and distortion. This duality, which Schacter refers to as memory's fragile power, is explored in an interwoven narrative of anecdote, case material and experimental findings. The result is a comprehensive, unique and engaging volume describing the facts and phenomena of human memory.

This is in fact a book like none other ever written about memory. There are more adjectives and adverbs than one finds in ordinary academic books, along with occasional appearances of the first-person singular. The book's illustrations, except one, are reproductions of contemporary art from the author's own collection. These features bring personal meaning to the topic of memory and make the book appealing and accessible to the general reader. Indeed, the popular readership is arguably the main target of the book. Yet working scientists, students and even the expert will find this book a valuable, carefully constructed and reliable resource. It is heavily annotated with 40 pages of notes and citations at the back, which serve as a companion to the

narrative text, and the bibliography fills an additional 35 pages. Both the main text and the notes are written with the even-handedness and critical eye of serious scholarship.

Experts may wonder at the studied neutrality with which Schacter discusses some issues that seem decidable on the basis of the evidence. Nevertheless, he is a gifted writer and a careful critic, and the nonspecialist could not find a more able guide to the extensive and sometimes

learned of the disaster, despite expressing high confidence in their recollections.

There is no evidence of improved accuracy of memory retrieval under hypnosis. Hypnosis increases the frequency of false recollections as much as the frequency of accurate ones. The elderly have weaker retentive memories than young adults, but, when asked to tell personal stories from their past, they produce narratives that are rated as more dramatic, more engaging and organized in a more complex way than those produced by younger people.

An important cause of inaccuracy in memory is the proneness to source amnesia, the misremembering of the who, when and where of a past event. A woman misidentified a psychologist she had seen on a live television interview as the man who attacked her on the same day. It is also possible to remember things that never

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UNAMABLE  
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REASONS

Anders Gunnar/Panos Pictures

**Total recall — rote learning of the Koran. Unfortunately, learning things parrot-fashion does not lead to improvement in the rest of one's memory.**

muddy literature on human memory. And, as an important contributor to the experimental science of human memory, Schacter is well positioned to lay out some of the fascinating, core facts about memory.

Recollection is not a literal replaying of the past but a reconstruction from fragments that can be influenced by the cues available at the time of retrieval. This key idea is easy to miss because it is not made concrete or linked to mechanism: that "the [retrieval] cue combines with the [memory trace] engram to yield a new, emergent entity — the recollective experience of the rememberer". Confidence and accuracy in recollection are not reliably correlated. College students interviewed 24 hours after the explosion of the space shuttle Challenger and again two-and-a-half years later often gave substantially different accounts on the two occasions of how they had

happened. Following ten weekly interviews with preschool children that encouraged them to think about both real and imagined events and to visualize the scenes, more than half of the children produced detailed and personalized 'memories' of a fictional happening, such as the time they had "a finger caught in a mousetrap and had to go to the hospital to get the trap off". Neuroimaging data indicate that imagined visual events are generated by some of the same brain systems that support visual perception.

Schacter provides a full treatment of conscious and nonconscious forms of memory, one of the most prominent topics in modern memory research. There is discussion of memory encoding, consolidation and retrieval; neurological and psychogenic amnesia; recent neuroimaging studies; and the characteristics of emotional memory. Finally, there is a

balanced discussion of the current debate over repression and recovery of memories of childhood sexual abuse.

Schacter's reading of the evidence of the last of these, altogether fair in my opinion, is that people may forget single abusive incidents and possibly multiple incidents but that persisting memory of abuse is more likely than forgetting. And when forgetting does happen, it is more likely to reflect normal memory processes, such as non-rehearsal, than some special, unconscious repression mechanism. Finally, if forgetting of extended abuse can occur, a dissociative disorder is more likely to be responsible than repression. With respect to the accuracy of recovered memories, accurate recollections can also occur, but illusory recollections can also occur and may be created inadvertently during psychotherapy; overall there is little scientifically credible evidence bearing on how often recovered memories are accurate, and no credible way at present to distinguish false recollections from veridical ones.

Memory's imperfections may be adaptive. Our species is good at accumulating knowledge — at inference, generalization and abstraction — not the literal retention of the individual events that lead to and support general knowledge. Freud wrote: "Normal forgetting takes place by way of condensation. In this way it becomes the basis for the formation of concepts." *Searching for Memory* is an effective and enlightening celebration of the diverse phenomena of human remembering, phenomena that will absorb the biological student of memory during the twenty-first century. □

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### Also remember...

**White Gloves: How We Create Ourselves Through Memory** by John Kotre. A professor of psychology provides a nontechnical survey of what cognitive science and clinical psychology have revealed about the capacities of human memory as well as its distortions and limitations. Free Press/Simon and Schuster, \$22, £15.99.

**Prospective Memory: Theory and Applications** edited by M. Brandimonte, G. O. Einstein and M. A. McDaniel. Contains 13 chapters on various aspects of 'prospective memory' (that is, our ability to remember what we must do), including encoding, storage and retrieval, ageing, neuropsychology and real-world applications. Lawrence Erlbaum, £53.50.

## Expecting the unexpected

Howard P. Segal

**Why Things Bite Back: New Technology and the Revenge Effect.** By Edward Tenner. Knopf/Fourth Estate: 1996. Pp. 346. \$26, £18.99.

DIFFER as they obviously do on the benefits and detriments of high technology, proponents such as Bill Gates and Alvin Toffler and opponents such as the neo-Luddite Kirkpatrick Sale and the alleged 'Unabomber' Theodore Kaczynski all agree that technological change is predictable. Whether the expected consequences be wonderful or dire, contemporary technology, both sides insist, will transform the world. The possibility that things may go awry rarely troubles such true believers in the power of technology.

To be sure, there is nothing new in their naive assumption that they can chart technology's future. Scores of earlier prophets and Jeremiahs have fallen victim to identical fantasies. Yet despite unprecedented access to information, the latest technological utopians and dystopians routinely ignore the readily available historical record of technology's mixed blessings. They are as shocked as their predecessors to discover one unanticipated development after another.

For these innocents above all, Edward Tenner's *Why Things Bite Back* should be required reading. With clarity, wit and considerable insight, Tenner offers abundant instances of what he terms 'revenge effects' — the unintended, ironic results of numerous mechanical, biological, chemical and medical improvements of this century. Revenge effects differ from side-effects: "If a cancer chemotherapy treatment causes baldness, that is not a revenge effect; but if it induces another, equally lethal cancer, that is a revenge effect".

Tenner's examples include new protective football equipment prompting such reckless behaviour as to make the game more dangerous than unprotected rugby; 'improved' (that is, non-wooden) tennis rackets leading to more boring games by accentuating power serves rather than exciting volleys; sturdier climbing gear encouraging more daring feats and in turn more injuries and deaths; low-tar, low-nicotine cigarettes discouraging smokers from quitting completely; widespread use of antibiotics generating drug-resistant microbes that are ever harder to combat; and repeated computer use intensifying back problems and carpal tunnel syndrome in hands and wrists. Other examples range from international shipping that brings rats to most of the

world's islands, to automobile alarm systems that increase car-jackings because frustrated criminals cannot readily steal the vehicles in conventional ways. The list goes on. The number of highly destructive South American fire ants multiplied rather than declined when DDT and related pesticides turned out to be more harmful to their predators. And inept testing of enhanced safety procedures caused the meltdown at the Chernobyl nuclear reactor.

*Why Things Bite Back* is no mere catalogue of 'revenge effects', however; nor is it a superficial scorecard of how twentieth-century technological advances veered off course. Not only does Tenner evince remarkably little scorn for those whose well-meaning inventions and schemes have backfired, but he also sympathizes with the rest of us who daily experience revenge effects and periodically recounts his own technological misadventures. Indeed, he says that his book began as he pondered why the 'paperless' computerized office like the one in which he then worked paradoxically required more and more paper to function efficiently. (Not surprisingly, he concludes that computers have failed to make offices and businesses more productive.)

Unlike many other critics and admirers of twentieth-century technology, Tenner never claims that the developments he details are unprecedented. Although one might wish for more historical context than Tenner provides, he does concede the existence of revenge effects throughout history. Such consequences have, of course, been illuminated by historians of technology. Tenner's point is that revenge effects are far more pervasive in this century than earlier just because of our false assumption that we have finally created 'fail-safe' systems in so many areas of work and leisure. Ironically, this overconfidence has led to an unprecedented need for vigilance to keep our complex systems working — and to repair them quickly when they inevitably break down. Moreover, where the old disasters were often 'spectacular' — such as a train wreck or a ship sinking — the new ones tend to be "diffuse, silent processes that continue almost invisibly", as is often the case with environmental pollution.

Tenner's title may at first glance give the impression that the things that bite back always do so deliberately and that, as in *Frankenstein* (1818) and its many imitators, technology out of control means technology personified. Yet Tenner, who discusses Mary Shelley's paradigmatic novel at the outset, never grants technology that degree of power over its creators. Even the microbes, animals and plants that have undermined our grand plans have done so in the natural, not the supernatural, scheme of things. As Tenner puts it, "[f]or most people, the prob-



lem is not open malice but repeated small episodes of frustration”.

Consequently, Tenner refuses to despair. He urges us, in effect, to expect the unexpected and to be more, not less, vigilant than our predecessors as we try to anticipate revenge effects and react to them when they do emerge. As he reminds us, Murphy's law — “if anything can go wrong, it will” — was intended by its originator, a frustrated military engineer, as a plea for alertness and adaptation, not a resignation to forces beyond our control. (So much for the intended effects of that declaration.) Tenner in fact offers a positive corollary: “Sometimes things can go right only by first going very wrong”. This optimistic view of disasters has been documented in several books by the civil engineer Henry Petroski, whom Tenner cites.

*Why Things Bite Back* offers a much-needed healthy balance between contemporary technological utopian fantasies and neo-Luddite despair. This superb guide to our high-tech world deserves a wide readership. □

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## Wishful thinking?

Massimo Piattelli-Palmarini

**The Logic of Failure: Why Things Go Wrong and What We Can Do to Make Them Right.** By Dietrich Dörner. Holt: 1996. Pp. 222. \$25.

MANY people, in many walks of life, are under the impression that things are not working as they should and that it is becoming more and more difficult to get some things, indeed anything, properly accomplished. Witness, for example, the hail of complaints in the international press about the dismal logistics and lapsed security of the Olympic Games in Atlanta. The problem, of course, is not to be found in the things themselves, but in the heads of the people who fail to carry them out. What has gone wrong? And what can be done about it?

Books on ‘why nothing works’ and ‘why we can't think straight’ make up a new literary genre, with diverse, at times perceptive, contributions by mind-reformers, film stars, policy-makers, educators, experts in managerial decision-making and professional psychologists and anthropologists. Explanations and recommendations range from a needlessly elaborate rephrasing of the obvious to genuinely novel psychological discoveries. The latest example is Dietrich Dörner's analysis of ‘decision traps’

VISIONS of the future as seen through the eyes of Americans earlier this century are explored by Joseph J. Corn and Brian Horrigan in *Yesterday's Tomorrows*, now out in paperback. Although technology was invariably seen to advance rapidly, society and politics were not — as witness plans for anti-aircraft flying circular-saws and waterproof furniture to allow “the housewife of 2000 [to] do her daily cleaning with a hose”. Originally published in 1984 to accompany a Smithsonian exhibition, the book contains a wealth of visual material drawn from popular science magazines, world trade fairs, films, advertisements and so on. Johns Hopkins University Press, \$24.95, £20.50.



in complex situations. Dörner, a professor at the University of Bamberg and director of the cognitive anthropology project at the Max Plank Institute in Berlin, has impeccable credentials in this special branch of cognitive psycho-anthropology. And he graces us with the nicest title so far: “The Logic of Failure”. It reflects his belief that “people court failure in predictable ways” and that “failure does not strike like a bolt from the blue; it develops gradually according to its own logic”.

His lively treatise, accessible to the cultivated lay reader, capitalizes on real-life cases (such as the Chernobyl disaster) and refined *ad hoc* experiments. In the latter, both expert and naive subjects are asked to make decisions in fictional, yet perfectly plausible, model worlds. Computers are then used to simulate the web of interlocking consequences of these decisions in the years ahead. The decision-makers have the benefit of absolute dictatorial power in changing the variables of the model, and can instantly check, if they so wish, the long-term consequences of any of their actions. The rationale for the approach is straightforward: “By removing the constraints of the real world, we hoped to see how people think and act when they are entirely free to do as they wish... [and] to illuminate the psychological factors bearing on human planning and decision making”.

So just how much has been learned

about these factors through such experiments? Despite many stimulating examples and judicious reconstructions of some recurrent decision traps, I am sorry to say that no new deep psychological discoveries have been made. Given a moment's reflection, most of us could predict what the faulty ingredients are likely to be: acting with insufficient reflection, short-sightedness in calculating consequences, becoming enamoured of our own predictions in the teeth of contradictory data, over-involvement with self-generated projects, excessive focus on small corners of a problem, neglect of early signals of impending catastrophe, and so on.

On these psychological mishaps, the book has, as one would expect, a lot to say; and what Dörner does say is lucid, well balanced, well supported and instructive, and invariably far more than just common sense. (I have witnessed top managers being fed with far lesser stuff on decision-making, in courses judged, to my amazement, to be quite successful.) But the reader will look in vain here for important insights comparable to those in other areas of the cognitive science of decision-making, such as the so-called ‘cognitive illusions’ pioneered by the findings of Daniel Kahneman and the late Amos Tversky. The academic discipline of ‘heuristics and biases’ that Kahneman and Tversky did so much to establish deals with deeply ingrained errors in decision-making under uncer-

tainty. It makes ample use of mathematics and is as 'hard' a science as anyone can hope to find in the elusive realm of mental mechanisms. In fact, it has even gained respectability in the world of pure economics and is routinely taught in the best business schools. All this is a far cry from the more traditional brands of the psychology of mistakes, such as that offered by Dörner.

What Dörner does mainly offer is a cogent plea for 'systemic thinking', for paying more attention to the connections, over and above the individual components. One of the problems is that in the complex scenarios modelled by Dörner there is no proper canon of normative sound decision-making. The discovery of cognitive illusions has taken place against the fertile background of inductive logic, probability theory, set theory, utility theory, the axioms of rational choice and other solid chunks of a theory of rationality. These normative canons do not explain why we recurrently fall prey to certain specific illusions: for that, we do need the experimental psychologist. But they allow us to identify on objective grounds which systematic mistakes we tend to make. It is not necessary to create complex scenarios to witness our dramatic decision traps; any garden-variety probabilistic judgement can show that we tend to fall into such traps. The logic of failure, it seems, is invariably tied up with some specific failure of our spontaneous logic. □

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## Signs of the time

Tom Kirkwood

**The Clock of Ages: Why We Age — How We Age — Winding Back the Clock.** By John J. Medina. Cambridge University Press: 1996. Pp. 332. £16.95, \$24.95.

ONCE upon a time, senescence was a privilege, the result of good fortune both in avoiding the ravages of diseases such as measles, diphtheria, typhoid, cholera, tuberculosis and pneumonia, and in escaping the multitudinous hazards of a harsh and often dangerous physical environment. Times have changed. Senescence is firmly on the agenda for most of us, first as individuals conscious of our own pending frailty, and second as members of a society undergoing a demographic revolution unparalleled in human history.

John Medina offers a tour of human ageing that aims to educate and enter-

tain. Popular science writing depends for its success on getting the right blend of content and style. Medina's style strikes a jarring note for those not keen on puns. Some truly nerve-jangling wordplay is displayed and even the title is a pun. The young Medina, we are told, used to torment his apparently much-loved mother by singing the title words of this book to the tune of the classic Christian hymn "Rock of Ages" while she contemplated her wrinkles in the mirror. Chapter subheadings such as "The eyes have it", "For whom the smell tolls" and "Hair today, gone tomorrow" are further examples of the dubious treats in store. The scientific text is leavened with anecdotes borrowed either from the author's early life or from the lives and, more particularly, the deaths of illustrious figures from the past. Billy the Kid, Florence Nightingale, Giovanni Casanova, Jane Austen and Napoleon Bonaparte all feature in this eclectic collection. Some of these anecdotes work and some do not, the oddest being a tale about army ants and a paraplegic scientist where the entire point of the story appears to have been omitted.

But what of the science? The book is structured in three parts dealing first with the broad biological picture, including comparative and evolutionary biology, then moving to an organ-by-organ view of human ageing, and closing with an examination of the mechanistic theories. Predictably the book ends with a discussion of scientists' views of life extension; and, equally predictably, the views that are quoted are biased towards the sensational.

Part one gropes its way towards a definition of ageing, a goal that the author candidly admits is hard to accomplish. The slippery quality of this definition was recognized in the early 1950s by Peter Medawar, who then attributed it to previous neglect, but some of the slipperiness still remains, despite the efforts of Medawar and later authors to grasp the matter more firmly. The problem arises from the diversity of species' life histories and the many layers at which senescence and death may be encountered within the organism. Nevertheless, considerable progress has been made, chiefly from the application of evolutionary life-history theory to the problem, and Medina fillets the issue more or less correctly.

Part two is well written and packs a great deal of human biology into eight chapters covering skin, hair, bones, muscles, joints, brain, heart, lungs, digestive system, senses and the reproductive system. Like an efficient butcher, Medina leaves little unaccounted for. The text is well-illustrated with diagrams and could serve as a useful primer for many purposes. The ageing dimension in each section is something of an add-on, however,

consisting of often no more than a paragraph or two under a subheading such as "How it ages".

An example of the somewhat limited penetration of the discussion on ageing in part two appears in the section on muscle. Muscle deterioration with age is attributed to loss of fibres, and three ideas are briefly mentioned to explain why this happens. One is through nerve death, leading in turn to inactivity, atrophy and death of muscle cells. The second is through age-related failure of the microvasculature. The third, briefly stated, is through breakdown in the mitochondria of muscle cells. An opportunity is missed here to discuss recent work on the mitochondrial theory of senescence, particularly as it concerns muscle. When eventually we get to mitochondria in more detail in a later chapter, Medina fails to mention the important point that mitochondrial changes happen most markedly during ageing of post-mitotic tissues, particularly muscle and brain. The hypothetical examples he mentions of cells affected by mitochondrial defects are from tissues with active cell turnover, where mitochondria alter much less with age.

The third part pits familiar opponents against one another. The 'stochastic damage' theories comprise one battalion of hypotheses proposing various types of random molecular damage as important contributors to senescence. These include oxidative damage, mutations, aberrant proteins and defective mitochondria. Medina reviews this camp adequately, and then lines up the so-called 'programme' theories against them. The programme theories are bolstered, at first sight, by various discoveries such as genes affecting rate of ageing in model systems such as the nematode *Caenorhabditis elegans* and premature ageing diseases in humans such as Werner's syndrome, as well as by the progressive uncovering of an intricate network of genes affecting cell division and programmed cell death. Shrewdly, Medina recognizes that this fight between programme and error theories is coming to seem increasingly artificial and that both kinds of mechanism have their place in the complex causality of human ageing.

So is it a good book or not? Like the proverbial curate's egg, it is good in parts. The science is adequate but too often fails to do real justice to this complex and challenging topic. The style is brisk and sometimes entertaining, although firmer editing for relevance would have helped. □

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# Chiron and the Centaurs: escapees from the Kuiper belt

Alan Stern & Humberto Campins

**The Centaurs—a group of objects orbiting chaotically among the giant planets of our Solar System—appear to be a population transitional in size between typical short-period comets and the large Kuiper-belt objects that orbit beyond Neptune. They promise to reveal much about the origin of and interrelationships between the icy bodies of the outer Solar System.**

THE outer Solar System has long appeared to be a largely empty place, inhabited only by the four giant planets, Pluto and a transient population of comets. In 1977 however, a faint and enigmatic object—2060 Chiron—was discovered<sup>1</sup> moving on a moderately inclined, strongly chaotic 51-year orbit which takes it from just inside Saturn's orbit out almost as far as that of Uranus. It was not initially clear from where Chiron originated.

Following Chiron's discovery, almost 15 years elapsed before other similar objects were discovered; five more have now been identified<sup>1</sup>. Based on the detection statistics implied by these discoveries, it has become clear that these objects belong to a significant population of several hundred (or possibly several thousand) large icy bodies moving on relatively short-lived orbits between the giant planets. This new class of objects, known collectively as the Centaurs, are intermediate in diameter between typical comets (1–20 km) and small icy planets such as Pluto (~2,300 km) and Triton (~2,700 km). Although the Centaurs are interesting in their own right, they have taken on added significance following the recognition that they most probably originated in the ancient reservoir of comets and larger objects located beyond the orbit of Neptune known as the Kuiper belt.

## Origin of the Centaurs

The first clue to the origin of the Centaurs came about as a result of dynamical studies of Chiron's orbit. At first discovered in the late 1970s<sup>3</sup>, and forcefully reiterated in more modern calculations<sup>4</sup>, Chiron's orbit is highly unstable to perturbations by the giant planets. As a result, Chiron's orbital lifetime among the giant planets is short, leading to the conclusion that its origin was in a more stable reservoir, either in the asteroid belt, or beyond the giant planets. The discovery of a coma around Chiron<sup>5–7</sup>, in the late 1980s, indicated the presence of surface volatiles which could not have survived the age of the Solar System in the comparatively warm asteroid belt<sup>8</sup>; such volatiles therefore strongly indicate that Chiron originated in a distant reservoir, beyond the giant planets.

A second line of evidence relating to Chiron's origin came about from simulations of cometary dynamics. These studies<sup>9–12</sup> demonstrated that the dominant dynamical class of short-period comets, called the Jupiter-family comets (JFC) cannot be derived from the classical, Oort-cloud cometary reservoir. The reason for this is that their characteristically low orbital inclinations cannot be efficiently produced by the action of planetary perturbations on orbits initially in the inclination-randomized (that is, nearly spherical) Oort cloud.

Instead, the JFC seem to derive from a dynamically stable reservoir concentrated near the plane of the planetary system. Any such reservoir for the JFC must satisfy the criterion that the loss rate of objects from it be low enough that the reservoir can persist for the age of the Solar System. Because the dynamical clearing time for orbits between the planets is characteristically one to two orders of magnitude shorter than the age of the Solar System<sup>12–14</sup>, there are few regions of space that provide stable,

candidate source locations for the JFC. One such region is the zone beyond Neptune's orbit (at 30 AU, astronomical units) where nonlinear perturbations by Neptune and the other giant planets can excite orbital eccentricities on timescales comparable to the age of the Solar System. Once orbital eccentricities are excited sufficiently to cause objects to cross Neptune's orbit, a fraction of these objects become temporarily trapped on Centaur-like orbits among the giant planets. Other such objects are dynamically transported by subsequent encounters with the other giant planets onto orbits that pass within 1–2 AU of the Sun<sup>15</sup>, where they generate comae and become easily detectable. A second possible source region for the JFC is the slowly dynamically evaporating jovian Trojan clouds, whose dynamics are controlled by the stability of a narrow phase space surrounding the leading and trailing lagrangian points of Jupiter. However, recent dynamical simulations<sup>16</sup> show that the jovian Trojan clouds are not as effective as the so-called trans-neptunian zone in populating the JFC and Centaur populations. Following historical suggestions dating back as far as the 1940s that a disk-shaped reservoir of planetesimals and other small objects might reside beyond the Neptune<sup>17,18</sup>, the trans-neptunian region has been dubbed the Kuiper belt, or in analogy to debris belts around the other stars, the Kuiper disk.

The pivotal breakthrough concerning the reality of the hypothesized Kuiper belt came in 1992, with the discovery of a faint (R-band magnitude near 23), 180-km diameter object<sup>19</sup> designated 1992QB<sub>1</sub>. 1992QB<sub>1</sub> orbits the Sun in a stable, nearly circular orbit some 14 AU beyond Neptune. In the four years since 1992QB<sub>1</sub> was found, over three dozen similar objects have been discovered in the trans-neptunian region<sup>2</sup>. Estimates<sup>2,20</sup> indicate that some of these objects have diameters approaching 400 km. Based on the efficiency with which such objects are being detected and their surface density on the sky, it has been estimated that around  $7 \times 10^4$  objects with diameters greater than 100 km orbit between 30 and 50 AU (ref. 2). Here we refer to these larger objects populating the Kuiper Belt as QB<sub>s</sub>.

Following the discoveries of numerous QB<sub>s</sub> in the Kuiper belt, the Hubble Space Telescope (HST) was used to conduct a search for the much smaller, and much fainter, cometary nuclei which must be present in this region if it is indeed a source of the JFCs. Last year, Cochran *et al.*<sup>21</sup> reported exciting evidence, near the limit of HST's capabilities, for numerous objects with V-band magnitudes of ~28.6, corresponding to comet-like diameters of a few kilometres to ~10 km. This evidence corresponds to a population of several hundred million comet-sized objects. If this result is coupled with models<sup>12</sup> that predict the ratio of the population detected in the region searched by Cochran to the entire trans-neptunian zone, a total population is calculated of several billion comets in the 30–50 AU region. As such, it appears that the Kuiper belt is indeed the source region of most JFC, as dynamical simulations predicted<sup>9,14</sup>.

Taken together, the discovery of both QB<sub>1</sub>- and comet-sized



objects in the Kuiper-belt region indicates that the Kuiper belt supplies a wide size range of objects onto orbits in the giant-planet region. Based both on expectations resulting from the planetesimal accretion codes, and the observational evidence for many more comets than QB<sub>1</sub>s, it appears that a power-law-like source population histogram exists in the Kuiper belt, with many more small bodies than QB<sub>1</sub>s. Because the dynamical transport process that brings objects from the belt to planet-crossing orbits is essentially independent of the mass of the object being transported<sup>15</sup>, it is expected that the population of objects ranging in size from Centaurs down to JFC orbiting among the giant planets is representative of the population of objects in the 30–50 AU zone from which they are derived.

### Physical attributes of the Centaurs

It is now established that the slow leakage of objects from the Kuiper belt due to planetary perturbations creates a population of objects on comparatively short-lived, planet-crossing orbits in the giant-planet region between 5 and 30 AU from the Sun. Studies of the dynamical evolution of orbits dislodged from the Kuiper belt<sup>15</sup> predict a characteristic equilibrium population of objects on planet-crossing orbits that is  $\sim 10^{-4}$  of the population of the Kuiper-belt reservoir from which they are derived. These studies also predict that the median lifetime of such orbits is of the order of  $5 \times 10^7$  years. Such findings imply that a population of  $\sim 5 \times 10^5$  to perhaps  $10^6$  comets, and  $\sim 30$ – $300$  Centaur objects of diameter 100 km or larger, are orbiting between the giant planets.

Chiron and its recently discovered cohort of Centaurs are thus now seen to be objects derived from the Kuiper belt. Table 1 summarizes the orbital attributes of the six known Centaurs; Fig. 1 depicts the orbits of these objects and their dynamical context in the outer Solar System.

As escapees from the Kuiper belt, the Centaurs are an important population for study. Indeed, owing to their greater proximity to the Sun, the brightest centaurs are some 5–7 astronomical magnitudes (factors of  $\sim 100$  to  $\sim 600$ ) brighter than the brightest

TABLE 1 Orbital characteristics of the known Centaurs

| Object              | Semi-major axis | Perihelion distance | Eccentricity | Inclination | Present opposition V magnitude |
|---------------------|-----------------|---------------------|--------------|-------------|--------------------------------|
| 2060 Chiron         | 13.70 AU        | 8.46 AU             | 0.38         | 25°         | 15.5                           |
| 5145 Pholus         | 20.30 AU        | 8.68 AU             | 0.57         | 7°          | 17.9                           |
| 1993HA <sub>2</sub> | 24.73 AU        | 11.84 AU            | 0.52         | 16°         | 21.0                           |
| 1994TA              | 16.82 AU        | 10.69 AU            | 0.31         | 5°          | 23.8                           |
| 1995DW2             | 25.03 AU        | 18.84 AU            | 0.25         | 4°          | 21.9                           |
| 1995GO              | 18.14 AU        | 6.79 AU             | 0.62         | 18°         | 20.3                           |

These characteristics are taken from ref. 53. Although the basic orbital properties of the third to sixth objects are known, they have not yet been named because, by IAU convention, objects must first be observed for long enough to produce astrometrically reliable orbits.

Kuiper-belt objects, which enables more detailed studies of the Centaurs than are possible with the QB<sub>1</sub>s and Kuiper-belt comets. Additionally, being closer to the Sun, the Centaurs experience greater heating, which generates characteristic perihelion surface temperatures in the range  $\sim 120$  to  $150$  K (ref. 22); by contrast, Kuiper-belt objects probably never experience surface temperatures in excess of  $60$ – $70$  K. Therefore, because vapour pressure depends exponentially on the temperature of the ice, Centaurs are much more likely than Kuiper-belt objects to show sublimation-generated activity. Although such heating causes the surfaces of the Centaurs to evolve chemically and physically over long time-scales<sup>23</sup>, it also causes the surface ices to sublime, and thus reveal valuable insights into the nature of these objects.

Unfortunately, although the Centaurs are brighter than Kuiper-belt objects, they are still faint in absolute terms, so considerable dedication is required to obtain physical information on them. As a result, comparatively little work has been done to reveal their compositions, colours, shapes, rotational properties and other attributes (Table 2). Despite the great deficits in our knowledge about the physical and chemical characteristics of this unique population, several important pieces of information are emerging.

First, with regard to the derived sizes of the Centaurs discovered to date, roughly half appear to be near 60 km in diameter, but 2060 Chiron<sup>22,24</sup> and 5145 Pholus<sup>25</sup> are much larger, with  $\sim 180$ -km diameters that are comparable to typical QB<sub>1</sub>s being discovered in the trans-neptunian zone. Second, infrared spectroscopy and colour photometry have given the first clues about the surface compositions of these objects. The first clearly detected spectral

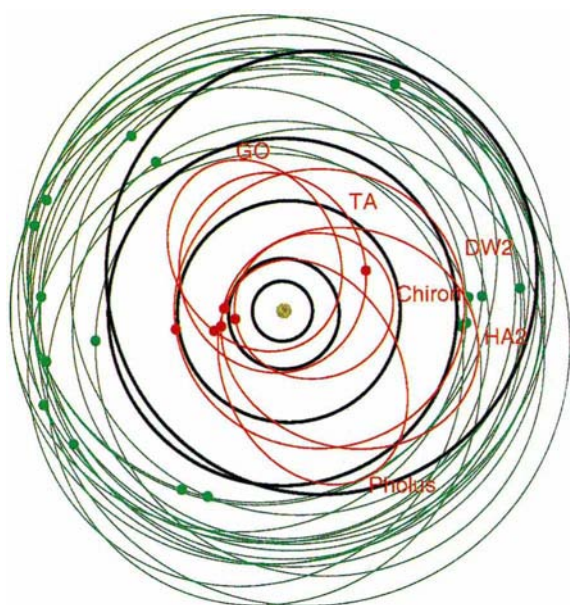


FIG. 1 The orbits of the giant planets (black lines), the six known Centaurs (red lines) and those Kuiper-belt objects with well established orbits (green lines). The dot on each orbit depicts the current location of the object. For scale, Jupiter's orbit is approximately 10 AU across. Abbreviations on the figure as follows: GO, 1995GO; TA, 1994TA; DW2, 1995DW2; Chiron, 2060 Chiron; HA2, 1993HA<sub>2</sub>; Pholus, 5145 Pholus. (Figure courtesy of H. F. Levison)

TABLE 2 Physical characteristics of the known Centaurs

| Object              | Diameter*<br>(km) | Geometric<br>albedo (in V) | Rotational<br>period | Rotational<br>amplitude | V - J colour‡ | Detected<br>activity |
|---------------------|-------------------|----------------------------|----------------------|-------------------------|---------------|----------------------|
| 2060 Chiron         | 182 ± 10          | 0.11–0.20                  | 5.92 h               | 9%                      | 1.13 ± 0.04   | Yes                  |
| 5145 Pholus         | 185 ± 22          | 0.04 ± 0.02                | 9.98 h               | 20%                     | 2.53 ± 0.06   | No                   |
| 1993HA <sub>2</sub> | 62 km†            | 0.05                       |                      |                         | 2.07 ± 0.40   | No                   |
| 1994TA              | 28 km†            | 0.05                       |                      |                         |               | No                   |
| 1995DW2             | 68 km†            | 0.05                       |                      |                         |               | No                   |
| 1995G0              | 60 km†            | 0.05                       |                      |                         |               | No                   |

\* The sizes of Chiron and Pholus were obtained<sup>22,24,25</sup> from thermal fluxes, with computed albedos based on their sizes and V magnitudes. Chiron's albedo is strictly an upper limit owing to a possible, small, residual coma contribution.

† These sizes were computed by assuming a V-band geometric albedo of 5%, a value which is commonly found for cometary surfaces.

‡ Colour data are discussed in the text<sup>27,31,32</sup>. Note a V - J colour of 1.116 would be identical to the Sun<sup>47</sup>, thereby indicating a neutrally coloured surface. Higher V - J colours indicate red surfaces.

absorption feature among the Centaurs was a deep 2.25- $\mu$ m absorption on Pholus<sup>26</sup>. This feature has been associated with light organic solids mixed with ices<sup>27,28</sup>. Importantly a 2.04- $\mu$ m absorption features has also now been detected, both in Pholus and Chiron<sup>29</sup>, which Cruikshank *et al.* identify<sup>30</sup> in Chiron as an absorption band of water-ice. Third, although none of the three Centaurs that have been explored in the infrared have displayed any statistically significant colour variation with rotational phase<sup>31</sup>, they do show striking colour differences between one another (refs 27, 32 and Fig. 2). Indeed, whereas Chiron's intrinsic colour is grey (that is, neutral) throughout the 0.3–2.5  $\mu$ m band, Pholus, which lies in a similar orbit and is similar in size, is extremely red. 1993HA<sub>2</sub>, though smaller and in a more distant orbit, is also very red compared to Chiron. How much of these differences between various Centaurs is due to evolutionary mechanisms (as opposed to intrinsic attributes) is not yet clear, but it is well established that long-term exposure to cosmic rays and solar ultraviolet radiation darkens (and initially reddens) surfaces containing light-weight organics, in turn creating a more complex chemical mélange.

Additionally, the determination of well constrained albedos for 2060 Chiron, and more particularly 5145 Pholus (because it is not active), provides useful information for predicting the sizes of QB<sub>1</sub>s from their observed magnitudes.

## The particular value of Chiron

Chiron is uniquely valuable among the Centaurs because of the long history of its sporadic outbursts. Why has such activity only been detected in Chiron? Possibly Chiron is dynamically younger, and therefore more active than the other objects. Alternatively the other objects may have thicker surface mantles, may be fundamentally different in their composition or simply may have not been observed long enough to expect to detect activity. Chiron's

uniqueness in showing activity is perhaps the most intriguing observable obtained on the Centaurs so far.

Chiron's activity was first recognized when it suffered an outburst that increased its brightness by a factor (in 1989) of just over two<sup>33,34</sup>. In 1989–91 Chiron was also observed to show a highly variable particulate coma and tail extending as far as  $2 \times 10^6$  km (refs 35, 36), and a cloud of CN gas<sup>37</sup> presumably derived from the photodissociation of some heavier, parent molecule. When these various observations were made, Chiron was still more than 10 AU from the Sun, where the solar radiation field is too weak to sublime water-ice, the common volatile that powers the cometary activity close to the Sun. Although other mechanisms remain plausible, the sublimation of highly volatile ices like CO, N<sub>2</sub> or CH<sub>4</sub> (buried a short distance below the surface) were therefore flavoured as the source of Chiron's activity. Further evidence for the sublimation of such volatiles was obtained through the discovery of even more extreme activity on archival, pre-discovery images of Chiron obtained when it was near its aphelion at 19 AU, and therefore far too cold to sublime anything but highly volatile ices like those mentioned above<sup>38</sup>. The final confirmation of this hypothesis came in 1995, though the discovery of CO gas itself in Chiron's coma<sup>39,40</sup>.

The fact that Chiron's activity was greater at its aphelion than it has been at any time since provides compelling evidence that its level of activity is not a simple function of heliocentric distance alone. Instead, Chiron's activity probably involves a complex interaction between the level of insolation reaching its surface, the obliquity of its spin axis, the location of its near-surface volatiles and extensive surface mantling by substances (possibly including silicates, water-ice and carbonaceous materials) which do not strongly sublime that far from the Sun.

Chiron's strong variability and the low gas-production rate inferred from CN and CO gas detections in its coma provide

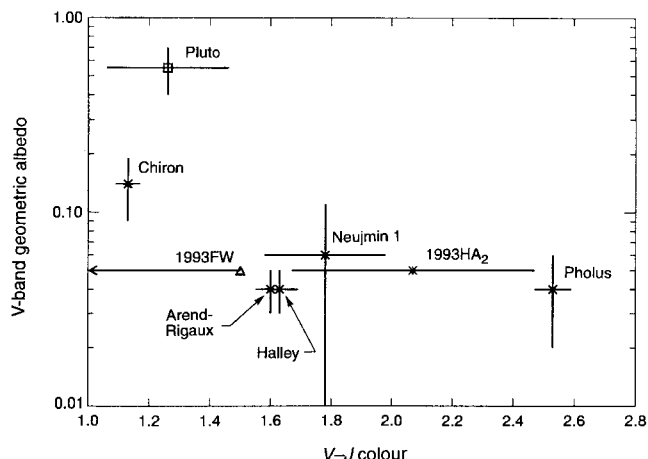


FIG. 2 Colour-albedo diagram showing the visible-band albedos and visible-infrared (that is, V - J) colours for several Centaurs (Chiron, Pholus and 1993HA<sub>2</sub>), several cometary nuclei (Arend-Rigaux, Halley and Neujmin 1), Pluto and the one QB<sub>1</sub> (1993FW) for which applicable data are available. A neutrally coloured surface would have the V - J colour of the Sun, 1.116 (ref. 54). Note that the Centaur Pholus is quite red; in fact, it is far redder than any other object in the Solar System. The V - J colour for 1993FW is an upper limit. V - J for Pluto was obtained from D. P. Cruikshank (personal communication; the V-albedo error bars for Pluto represent its intrinsic rotational lightcurve variation).

strong evidence that Chiron's activity is generated by localized sources covering  $<1\%$  of the surface. The case supporting highly localized vents or jets on the surface is further supported by short-term brightness fluctuations in Chiron's coma<sup>41,42</sup> that occur on timescales consistent with clouds of material being ejected onto suborbital trajectories, and by the detection of complex opacity structures in Chiron's coma during two recently observed stellar occultations by Chiron<sup>24,43</sup>. It has been pointed out<sup>44</sup> that these vents or jets may resemble the geysers detected on the surface of Neptune's large, captured satellite, Triton.

Chiron's low gravity creates a situation in which its escape speed ( $\sim 10^2 \text{ m s}^{-1}$ ) is comparable to both the thermal velocity of sublimating gas ( $\sim 2 \times 10^2 \text{ m s}^{-1}$ ), and the estimated muzzle velocity of Triton-like geysers<sup>45</sup> (of the order of  $40\text{--}300 \text{ m s}^{-1}$ ). As a result, some of the gas and entrained particulates ejected from Chiron's surface would be deposited into high suborbital trajectories; much would also escape. Modellers have only begun to explore the range of interesting physical phenomena likely to result in this intermediate regime between freely escaping cometary comae and strongly bound planetary atmospheres<sup>46</sup>. Among these is the distinct possibility that Chiron's neutral colour and comparatively high albedo are the direct result of its activity, which probably causes a thin veneer of icy particulates to rain back onto the surface from suborbital trajectories.

## An emerging view

We are witnessing a revolution in our understanding of the content and architecture of the outer Solar System. Whereas a decade ago, the outer Solar System seemed to consist only of the outer planets, the Oort-cloud comets and the then-rookie object Chiron, we now see revealed both the teeming Kuiper belt and its progeny, the comets and Centaur-sized objects orbiting among the outer planets. As a result, we have come to recognize that the

outer Solar System is littered with icy objects intermediate in size between comets and the giant planets.

We have also learned that the early stages of planetary formation, with widespread growth from planetesimals to objects with diameters of several hundreds of kilometres, provide concrete evidence for an ancient era of planet-building in the Kuiper-belt region<sup>47,48</sup>. For some reason (probably involving the role of Neptune that excited orbital eccentricities that were not conducive to further growth), the era of accretion in the Kuiper belt was prematurely truncated at a stage where intermediate-sized objects had formed<sup>49</sup>. The strong circumstantial evidence for the early formation of numerous objects in the 1,000-km class, of which Pluto and Triton are apparently the sole extant remnants within observational reach<sup>50,51</sup>, further supports the case for initially strong but eventually arrested planetary accretion in the Kuiper-belt region<sup>48,52</sup>. As such, the Kuiper belt has become one of the most important regions in the Solar System for studies of planetary origins. The Centaurs and QB<sub>1</sub>s therefore represent a valuable, relic population of icy objects whose growth was arrested at a fascinating, intermediate stage between comets and small planets.

The Centaurs also serve as bright proxies for distant comets, as laboratories for studying surface processes occurring on comets, Triton and perhaps Pluto, and as nearby proxies for the QB<sub>1</sub>s and other intermediate-scale bodies that bridge the size gap between comets, Pluto and Triton. As such, they hold special promise for understanding the origin and interrelationships among the icy bodies of the outer Solar System.  $\square$

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# Cdc25 cell-cycle phosphatase as a target of c-myc

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**The product of the proto-oncogene c-myc, in partnership with Max, forms a transcription factor that can promote either oncogenic transformation or apoptosis. The Myc/Max heterodimer binds to elements in the *cdc25A* gene and activates transcription. Like *myc*, *cdc25A*, itself a proto-oncogene, can induce apoptosis in cells depleted of growth factor, and Myc-induced apoptosis also requires *cdc25A*. These findings indicate that *cdc25A* is a physiologically relevant transcriptional target of c-myc.**

The c-myc proto-oncogene belongs to a family of related genes implicated in the control of normal cell proliferation and the induction of neoplasia<sup>1-4</sup>. The first identified member of the family, v-myc, was found as a transforming gene of the MC29 and MH2 avian retroviruses<sup>1-4</sup>. A cellular homologue was later identified<sup>5</sup> and isolated<sup>6</sup>. c-myc expression is associated with mitogenic activation in many cell types<sup>7,8</sup>. Oncogenic activation of the gene in non-virally infected cells results from constitutive over-expression, often caused by gene amplification and chromosomal translocation<sup>9,10</sup> but not generally from mutation in the c-myc coding sequence<sup>9,10</sup>. More recently, c-myc has been implicated in the induction of apoptosis, or programmed cell death<sup>11,12</sup>. Myc-induced apoptosis is enhanced by growth-factor deprivation, and the oncogenic-apoptotic switch is sensitive to the level of mitogenic stimulation<sup>11,12</sup>.

c-myc protein (Myc) is a transcription factor<sup>9,13-27</sup> that acts in conjunction with its partner, Max<sup>16-27</sup>. Few transcriptional targets of Myc have been identified<sup>28</sup>. One, ornithine decarboxylase, is an essential enzyme involved in the synthesis of polyamines<sup>29</sup>. Ornithine decarboxylase displays certain oncogenic properties, suggesting that it might be an important Myc target<sup>28,29</sup>. Induction of *myc* in growth-factor-depleted cells causes activation of the cyclin E/Cdk2 kinase without altering the abundance of Cdk2 or cyclin E (ref. 30). In this situation, although Cdk2 is inactive, it is phosphorylated at the essential Thr 160 residue before *myc* induction, suggesting that activation of Cdk2 may be mediated either by dephosphorylation of the Thr 14 or Tyr 15 residues, or by inactivation of protein inhibitors such as p21, p27 or p16.

In human cells, there are three known CDK-activating phosphatases, Cdc25A, B and C<sup>31,32</sup>. Among these, *cdc25A* is expressed early in the G1 phase of the cell cycle following serum stimulation of quiescent fibroblasts<sup>33</sup>. *cdc25B* is expressed closer to the G1/S transition<sup>34</sup>, and *cdc25C* is activated in G2 (ref. 31). *cdc25A* and *cdc25B* cooperate with Ha-ras in the oncogenic transformation of primary rodent fibroblasts<sup>35</sup>, and both *cdc25B*<sup>35</sup> and *cdc25A* (M. Loda, personal communication) are overexpressed in certain primary breast tumours. In combination, these observations prompted us to evaluate whether human *cdc25* genes might serve in either oncogenesis or apoptosis as direct transcriptional targets of c-myc.

## ***cdc25* expression stimulated by Myc**

To determine whether *cdc25* genes might be transcriptionally activated by Myc, we used previously described cell lines (LM3<sup>36</sup> and Val5<sup>37</sup>) expressing a Myc-oestradiol-receptor fusion protein (Myc-OR)<sup>36,37</sup> that is activated by the addition of  $\beta$ -oestradiol to the growth medium<sup>38</sup>. Activation of myc-OR in serum-deprived

cells causes induction of *cdc25A* gene expression (Fig. 1a). An increase in *cdc25A* messenger RNA was consistently detected by 3–4 hours after addition of oestradiol, peaking after 8–16 h at a level four times higher than that found in uninduced cells (Fig. 1a). A twofold induction of *cdc25B* was also observed at the 8-hour time point (Fig. 1a). The same blots were reprobbed with  $\beta$ -actin complementary DNA to ensure that RNA loading in each lane was roughly equal (Fig. 1a). The level of the *cdc25C* message is low in LM3 cells and did not change with oestradiol treatment (data not shown). Similar results were obtained using Val5 cells (data not shown).

To investigate further the specificity of stimulation of *cdc25A* expression by Myc, we treated LM3 cells either with  $\beta$ -oestradiol or with hydroxytamoxifen (HO-TM), a synthetic compound that stimulates activity of the Myc-OR fusion protein but is inactive against direct  $\beta$ -oestradiol gene targets<sup>30</sup>. This experiment was also done in the presence or absence of the protein synthesis inhibitor cycloheximide (Fig. 1 legend). In LM3, both  $\beta$ -oestradiol and HO-TM activate *cdc25A* expression. Cycloheximide fails to inhibit activation, indicating that induction of *cdc25A* by Myc-OR does not require new protein synthesis. No stimulation was observed with cycloheximide alone (Fig. 1b).

We next determined whether the abundance of Cdc25A protein increased in parallel with mRNA. We used LM3 cell extracts, prepared before and after stimulation with  $\beta$ -oestradiol to determine the abundance of the Cdc25A protein by immunoblotting. An increase in the level of Cdc25A was observed as early as five hours after induction of Myc-OR with  $\beta$ -oestradiol (Fig. 1c). Antigenic peptide competition completely inhibited immunoprecipitation of the 67–72K Cdc25A protein.

Finally, we investigated whether Cdc25A protein could be induced by a non-oncogenic mutant of *myc*. A deletion mutant, Myc $\Delta$ 105–143, shows no oncogenic or apoptotic activity<sup>12,38</sup>. We used Rat1 cells expressing Myc-OR or (Myc $\Delta$ 105–143)OR<sup>39</sup>, to compare the level of *cdc25A* expression following stimulation by  $\beta$ -oestradiol. The basal level of Cdc25A is higher in serum-starved cells expressing Myc-OR than in those expressing  $\Delta$ Myc-OR. Cdc25A is further induced by  $\beta$ -oestradiol in Myc-OR but not in  $\Delta$ Myc-OR cells (Fig. 1d).

## **Myc/Max binding sites**

To investigate whether the regulation of *cdc25* by Myc might be direct, we used a modified precipitation assay to search for Myc/Max binding sites genomic human *cdc25A*, *B* and *C* DNA clones (Fig. 3 legend). Briefly, *cdc25A* (12E), *cdc25B* (17K) and *cdc25C* (15A) genomic clones were digested with *Hinf*I and subjected to affinity chromatography on glutathione-S-transferase (GST)–Myc/Max

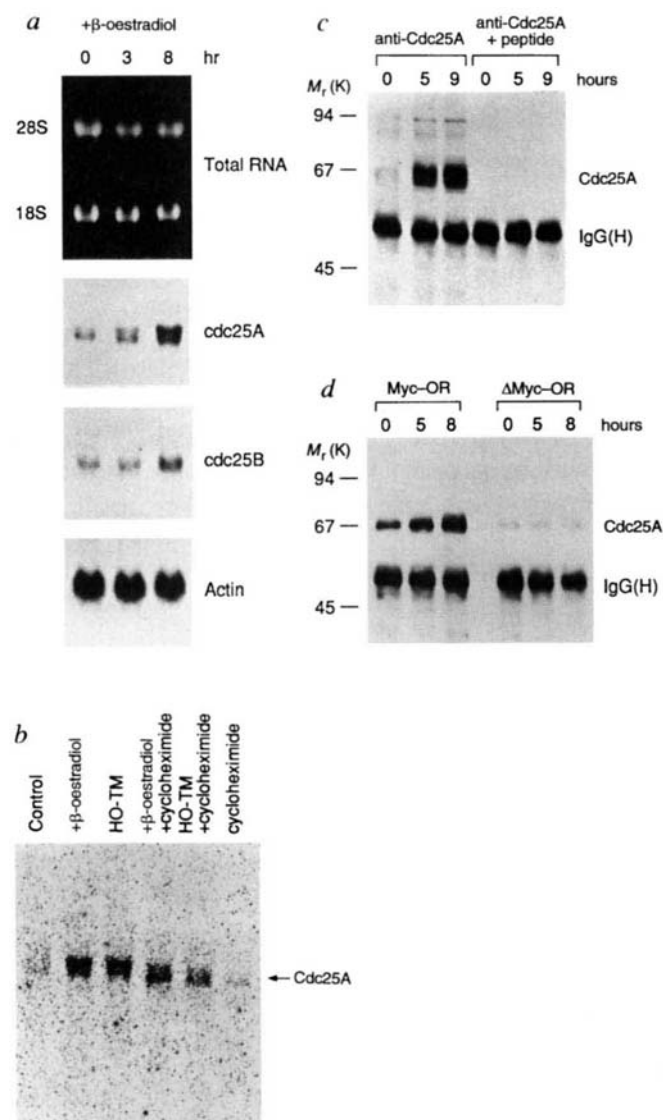


FIG. 1 Stimulation of *cdc25* expression by c-myc. **a**, Northern blot showing *cdc25A*, *cdc25B* and actin expression in a cell line (LM3) expressing Myc-oestradiol-receptor fusion protein (Myc-OR) on stimulation with  $\beta$ -oestradiol for the indicated times. 28S and 18S ribosomal RNA markers are indicated. **b**, Northern blot showing *cdc25A* expression in LM3 cells following treatment with  $\beta$ -oestradiol or hydroxytamoxifen (HO-TM) in the presence and absence of 10  $\mu$ M cycloheximide or cycloheximide alone. **c**, Western blot analysis of Cdc25A level upon stimulation of LM3 cells with  $\beta$ -oestradiol for the indicated time. + peptide, the antibody was preincubated with its cognate peptide antigen before immunoprecipitation<sup>48</sup>. **d**, Western blot of Cdc25A in Rat1 cells, expressing wild-type Myc-OR or mutant Myc( $\Delta$ 105–143)-OR fusion proteins. Cells were treated with  $\beta$ -oestradiol for the indicated times.

**METHODS.** Mouse cell lines expressing Myc-OR fusion protein<sup>36,37</sup> were provided by N. Hay and H. Hermeking; Rat1 cells expressing Myc-OR and Myc( $\Delta$ 105–143)OR were from L. Penn<sup>39</sup>. Cells were grown in DMEM (without phenol red) containing 10% fetal calf serum (Sigma), incubated for 48–72 h in DMEM with 0.1% fetal calf serum and then stimulated by addition of  $\beta$ -oestradiol to 1  $\mu$ M. For **b**, cells were treated either with 1  $\mu$ M  $\beta$ -oestradiol or 0.2  $\mu$ M HO-TM alone, oestradiol or HO-TM with 10  $\mu$ M cycloheximide or cycloheximide alone. Cells were collected at the indicated times (15 h in **b**), RNA was isolated using Trizol (Gibco/BRL). 10  $\mu$ g total RNA from each time point was loaded onto a 1% agarose gel. Northern blot analysis was done using a 1.6-kb *NotI* fragment of *cdc25A* (ref. 35), corresponding to the complete open reading frame, 1.2-kb *NotI*/*EcoRI* fragment of the *cdc25B* or human  $\beta$ -actin probe. For **c** and **d**, cells were lysed in immunoprecipitation buffer<sup>48</sup> and Cdc25A immunoprecipitated as described<sup>48</sup>. Western blot analysis was done using antibodies against the GST-Cdc25A fusion protein.

complexes (Fig. 3 legend). Two DNA fragments (F1 and F2) from the *cdc25A* genomic clone consistently bound to Myc/Max complexes (Figs 2a, 3a). No binding was observed with sequences from the *cdc25C* genomic clones and a single fragment was detected in the *cdc25B* genomic clone (data not shown). Nucleotide sequencing revealed that F1 and F2 mapped to the first intron of the human *cdc25A* gene (Fig. 2a) and that within each fragment there was a single site that matched the Myc consensus binding motif (MB1 and MB2; Fig. 2a, b). Subsequently, the genomic *cdc25A* clone (12E) was fully sequenced and revealed a third potential Myc-binding site in the second intron, MB3 (Fig. 2a, b).

To test whether Myc/Max complexes could efficiently bind to the isolated fragments, we did electrophoretic mobility shift assays (EMSA) on F1 and F2, confirming that they represent functional Myc-binding elements *in vitro*. An example of such an assay with F1 is shown (Fig. 3b). An oligonucleotide, matching the Myc-binding site from F1 (MB1; Fig. 2a) was also tested by EMSA (Fig. 3c). In this case, double-strand oligonucleotides corresponding to the putative wild-type (WT31) F1 Myc-binding site or mutant (MUT31) Myc-binding site (Fig. 3 legend) were used to compete with end-labelled WT31 (Fig. 3 legend). Efficient binding, indicated by changes in electrophoretic mobility of WT31, was observed with Myc/Max, but not with Myc alone. This was competed by the wild-type but not by the mutant binding site (Fig. 3c). Similar results were obtained with MB2 and MB3 sites (not shown).

## Transcriptional activation

To establish whether the observed Myc/Max binding elements might act as functional enhancers, a variety of reporter constructs were prepared. We subcloned the 2.2-kilobase(kb) *KpnI*/*HindIII* fragment from the genomic *cdc25A* clone containing the MB2 and MB3 binding sites (Fig. 2a), the 0.7 kb *SacI* fragment containing MB3 (Fig. 2a), the F2 *HinfI* fragment containing MB2 (Fig. 2a), and the *HinfI* F1 fragment containing MB1, into a construct that drives luciferase-gene transcription under control of the SV40 minimal promoter (Fig. 4 legend; pGL3 promoter from Promega). In addition, a 27-base-pair oligonucleotide comprising minimal MB3 (Fig. 2b) was introduced in one, two or three copies into the same vector. Transient co-transfection assays using the luciferase-reporter constructs and a Myc-expressing plasmid, pM21 (ref. 13), were done in NIH3T3 cells. The relative activation of each reporter construct by Myc is shown in Fig. 4a. The level of activation is broadly similar to that described for other Myc-binding elements and is consistent with the level of *cdc25A* induction by Myc-OR *in vivo* (ref. 40 and Fig. 1a).

We also systematically scanned the *cdc25A* genomic clone for functional Myc-responsive elements in the context of the natural *cdc25A* promoter. *SacI* fragments spanning the entire 5.6-kb *cdc25A* genomic clone (Fig. 2a) were subcloned upstream of the 0.5-kb *BamHI*/*XhoI* fragment of the *cdc25A* genomic clone. This is the region between –400 and +108 relative to the first nucleotide of the known cDNA sequence of *cdc25A* and referred to as the natural *cdc25A* promoter (NP series; Fig. 4 legend). Transient transfection experiments with these constructs demonstrate 4–5-fold Myc-dependent transcriptional activation with the 0.7-kb *SacI* fragment containing the MB3 site (Fig. 4b) and little activation with the other *SacI* DNA fragments (data not shown). Interestingly, the MB1 element responded to Myc in the context of the F1 fragment (Fig. 4a) but not the 2.8-kb *SacI* fragment (data not shown), perhaps indicating dependency of MB1 function upon distance from the *cdc25A* promoter or, alternatively, the presence of negative regulatory elements within the 2.8-kb *SacI* fragment.

To test further the role of the putative Myc-responsive element within the 0.7-kb *SacI* fragment, we performed site-directed mutagenesis, changing CACGTG to CACCTG in the MB3 region (Fig. 3a). This mutation completely abolished transcriptional activation by Myc (Fig. 4b). Similar results were obtained by mutation of MB1 within F1 (data not shown). To find out whether MB3 could activate transcription when positioned further away

from the promoter, at a similar distance to that between MB3 and the promoter in *cdc25A*, we introduced the 0.7-kb fragment containing MB3 or a mutant (CACCTG) MB3 fragment downstream of luciferase (Fig. 4b, methods). We found that the wild-type but not the mutant MB3 was responsive to Myc in this context (Fig. 4b). Thus, MB3 was completely functional within a circular plasmid at a distance of 2.5 or 3.5 kb relative to promoter in a distant enhancer position. The ability to activate transcription

from a distant enhancer position discriminates between Myc/Max and other transcriptional factors capable of interaction with E-boxes<sup>41</sup>.

Finally, we investigated which domains of the Myc protein are required for transcriptional stimulation of *cdc25A*. Myc constructs with deletions in either the transcriptional-activation ( $\Delta 7-91$ ), helix-loop-helix ( $\Delta 371-410$ ), or leucine-zipper domains ( $\Delta 414-433$ ) were inactive in transcription activation (Fig. 4c), suggesting

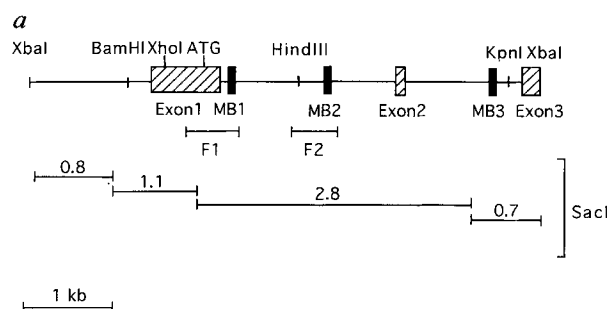


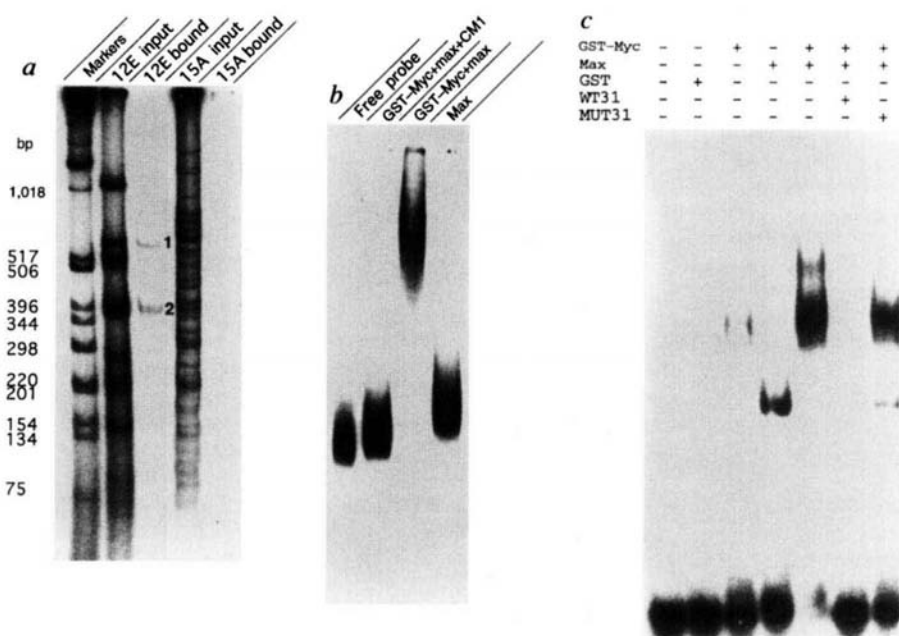
FIG. 2 Map of the human *cdc25A* gene. *a*, Map of the human genomic *cdc25A* clone (12E). MB1, 2 and 3 show positions of the putative Myc-binding sites. F1 and F2 correspond to the Myc/Max-binding *HinfI* restriction fragments, which were identified in a modified precipitation assay (Fig. 3 legend). The positions of 0.7-, 0.8-, 1.1- and 2.8-kb *SacI*

|       |          |               |               |
|-------|----------|---------------|---------------|
|       | - 8      |               | + 18          |
| MB1   | GGGCCTGC | <b>CATGTG</b> | CACCCGCCCCGG  |
| MB2   | GCACAAGA | <b>CATGTG</b> | CTTAGCAACGGG  |
| MB3   | ACTACAGA | <b>CACGTG</b> | CCACCACACCAA  |
| ODC1  | TGTGCGGC | <b>CACGTG</b> | TCGCGAGGCCCG  |
| ODC2  | GCAGGGGA | <b>CACGTG</b> | GTCCCGGAGCNG  |
| PT    | GCAACGAG | <b>CACGTG</b> | GCCTGGGGCGCCA |
| Cons. | C C G    | <b>CACGTG</b> | CC A CC G     |
|       | G G      | <b>T</b>      | GT C G A      |

fragments are indicated. Striped boxes correspond to exons of the *cdc25A* gene. *b*, Comparison of the Myc/Max binding sites within the *cdc25A* (MB1, MB2 and MB3), prothymosin (PT)<sup>40</sup> and ornithine decarboxylase (ODC)<sup>29</sup> genes. Cons, consensus

FIG. 3 *a*, Identification of Myc/Max binding sites in the human genomic *cdc25A* clone. Size markers (bp) are indicated. 12E input, end-labelled 12E (*cdc25A*) genomic *HinfI* fragments (see Methods); 12E bound, end-labelled 12E fragments bound to GST-Myc/Max complexes. 1 and 2 correspond to F1 and F2, respectively (see Fig. 2a and text); 15A input, end-labelled 15A (*cdc25C*) genomic *HinfI* fragments (see Methods); 15A bound, end-labelled 15A *HinfI* fragments bound to GST-Myc/Max. *b*, Electrophoretic mobility-shift assay (EMSA) with end-labelled F1 fragment (Fig. 2a) alone (free probe), in the presence of Myc/Max complexes and an excess of competing oligonucleotide<sup>25</sup> (Myc/Max + CM-1), in the presence of Myc/Max complexes (Myc/Max) or in the presence of Max alone (Max). CM-1 indicates a high-affinity c-myc-binding double-strand oligonucleotide<sup>25</sup>. *c*, Myc/Max and Max interaction with a synthetic oligonucleotide (WT31) consisting of the MB1 sequence (Fig. 2b). Purified GST-Myc, Max or GST proteins and unlabelled WT31 or MUT31 were added as indicated (MUT31 is WT31 with a mutation of CATGTG to CAACTG).

**METHODS.** To identify Myc/Max-binding sequences in the *cdc25* genomic regions we used affinity chromatography, similar to previously described immunoprecipitation procedures. Briefly, *cdc25A*, *B* and *C*) genomic clones (12E, 17K and 15A; provided by D. Demetrick) were digested with the *HinfI* restriction endonuclease, end-labelled with <sup>32</sup>P, incubated with 10 ng of the GST-Myc/Max complexes in binding buffer<sup>25</sup> for 10 min at 4 °C. 20  $\mu$ l glutathione-Sepharose was added and incubation continued for 30 min at 4 °C. Isolated complexes were washed four times in binding buffer, eluted by proteinase-K digestion, treated with phenol/chloroform and ethanol-precipitated. Precipitated material was resuspended in 10–20  $\mu$ l of 10 mM Tris-HCl, 1 mM EDTA, pH 7.6 (TE) and subjected to polyacrylamide gel electrophoresis, followed by autoradiography. Total digests were treated in the same way, except for affinity chromatography, and run as a control in a parallel lane with purified



material. Electrophoretic mobility-shift assays were done either with the F1 fragment or with the double-stranded oligonucleotide corresponding to MB1 as described<sup>25</sup>. The exact sequence of the wild-type oligonucleotide (WT31) matches MB1 (Fig. 2b), with the addition of two cytidines at the 5' and 3' ends. The mutant oligonucleotide in *c* (MUT31) has the same sequence as WT31, except that CATGTG was changed to CAACTG. Briefly, double-stranded WT31 oligonucleotide was labelled with <sup>32</sup>P using polynucleotide kinase, incubated in the presence of GST, GST-Myc, or GST-Myc/Max mixture and subjected to EMSA as described<sup>25</sup>. Similar experiments were repeated using oligonucleotides matching MB2 and MB3 as shown in Fig. 2b. The mutant oligonucleotide corresponding to MB2 had CAACTG in place of CATGTG, and the mutant oligonucleotide corresponding to MB3 had CACCTG in place of CACGTG (Fig. 2b).



that these domains, known to be required for Myc activity, are also important for *cdc25A* transcriptional activation.

### Cdc25A role in apoptosis

Physiologically relevant *c-myc* targets might be expected to promote two seemingly contradictory cellular responses, cell-cycle activation and apoptosis. To investigate whether *cdc25A* might trigger apoptosis, we introduced the gene, under a tetracycline-repressible promoter, into 3T3 LI cells that undergo Myc-dependent DNA replication and apoptosis in growth-factor-depleted media<sup>36</sup>. We isolated two independent cell lines (A10 and A12) which increase *cdc25A* protein levels ~4-fold in response to tetracycline removal from the medium (Fig. 5A). In normal growth media, A10 cells continue to proliferate regardless of *cdc25A* induction (data not shown). As Myc causes apoptosis in cells that are largely depleted of growth factors<sup>12,36,37</sup>, we incubated A10 cells for 48 h in medium containing 0.1% serum before removing tetracycline. Eighteen hours later, significant cell death was seen, and at 36 h 40–50% of cells had died. Control cultures incubated for the same period in tetracycline-supplemen-

ted medium showed no significant apoptosis (not shown). To confirm that the observed cell death was by apoptosis, we used TUNEL assays (Fig. 5C). Upon *cdc25A* induction, cells stained positively (shown at 18 and 36 h post-induction in Fig. 5C *a* and *b*), in parallel with morphological changes, which were more pronounced at 36 h (compare Fig. 5C *d* and *e*). In addition, apoptosis in A10 cells was associated with the development of a typical ladder of DNA degradation (Fig. 5B).

We next investigated whether apoptosis caused by *cdc25* expression was dependent on the status of p53, we introduced either *cdc25A*, *cdc25B* or *cdc25C* into 3T3 LI, 3T3 BALB/c and p53<sup>-/-</sup> fibroblasts in a transient transfection assay (Fig. 5D). In three independent experiments, *cdc25A* efficiently induced apoptosis in 3T3 LI cells. *cdc25B* was less potent, and no apoptosis was observed upon introduction of *cdc25C* (Fig. 5D). Myc-driven apoptosis is greatly reduced in cells that have mutant p53 (refs 36, 37). We find that, in contrast to 3T3 LI cells, which carry wild-type p53, 3T3 BALB/c and p53<sup>-/-</sup> fibroblasts (both lacking p53 function) support Cdc25A-induced apoptosis very poorly (Fig. 5D).

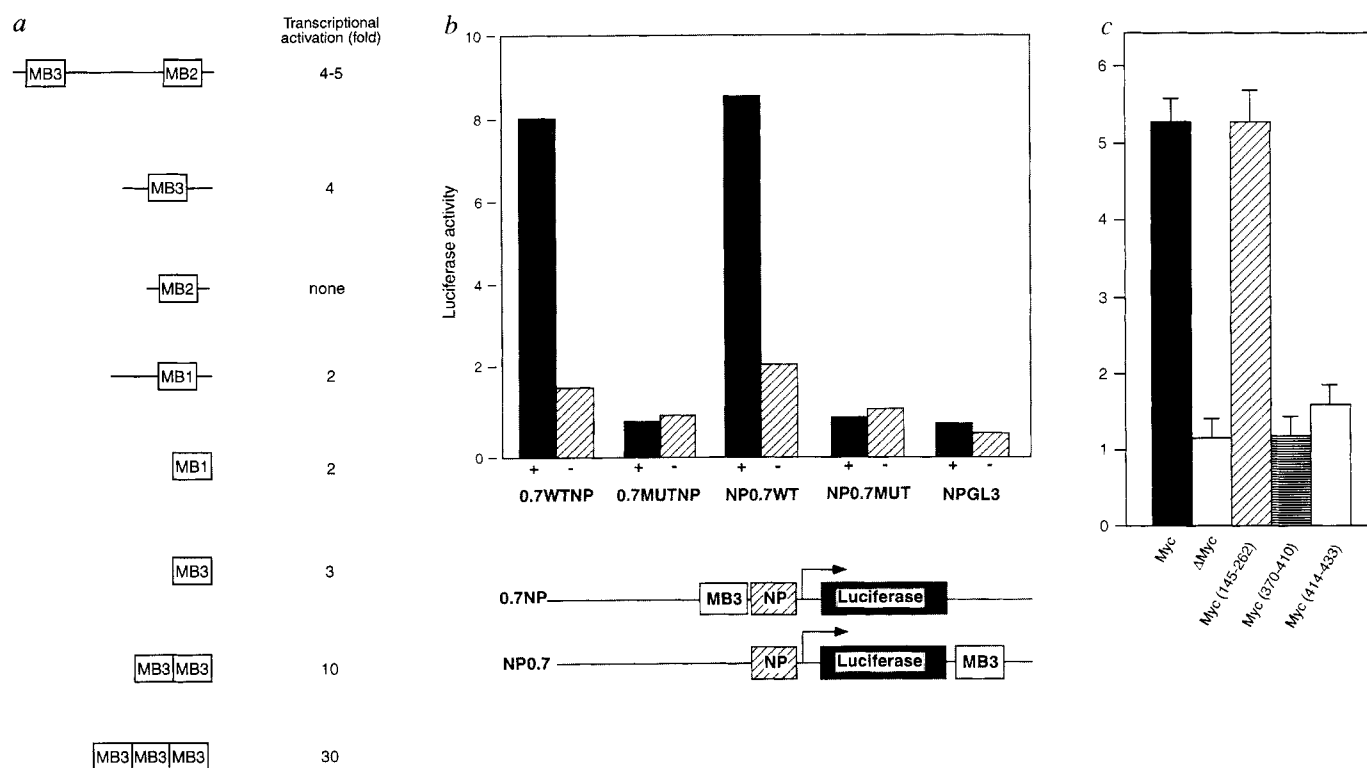


FIG. 4 Myc-dependent transcriptional activation. *a*, Genomic fragments tested for Myc-dependent transcriptional activation of a luciferase-reporter plasmid (pGL3). From top to bottom: 2.2-kb *KpnI/HindIII* fragment (Fig. 2a), 0.7-kb *SacI* fragment (Fig. 2a), 0.37-kb F2 fragment (Fig. 2a), 0.55-kb F1 fragment (Fig. 2a), 27-bp oligonucleotides, corresponding to the MB1 or MB3 sites as shown in Fig. 2b, two copies of MB3 site, and three copies of MB3 site in pGL3 promoter plasmid. The relative level of transcriptional activation comparing luciferase activity in the presence and absence of Myc is shown. *b*, Myc-dependent transcriptional activation (in arbitrary units) of the pGL3NP reporter alone (NPGL3; see Methods) or pGL3NP with the wild-type (WT) or mutant (MUT) 0.7-kb *SacI* fragment located upstream (0.7WTNP, 0.7MUTNP) or downstream (NP0.7WT, NP0.7MUT) of the natural promoter. Luciferase activity in the presence (+, filled boxes) or absence of Myc (–, hatched boxes) in relative light units is shown. *c*, Transcriptional activation of the pGL3NP reporter containing the MB3 site by *c-myc* and *c-myc* mutants; luciferase activity in relative light units is shown.

METHODS. *Cdc25A* genomic fragments, containing MB2 and MB3 (2.2-kb

*KpnI/HindIII* fragment; Fig. 2a), MB1 (0.55-kb F1 fragment; Fig. 2a), MB2 (0.37-kb F2 fragment; Fig. 2a), MB3 (0.7-kb *SacI* fragment; Fig. 2a) and single, double or triple copy of the 27-bp MB3 site (Fig. 2b) or single copy of the MB1 site (Fig. 2b) were subcloned into pGL3Promoter plasmid (Promega) and pGL3 with the natural *cdc25A* clone (Fig. 2a). The 0.7-kb *SacI* fragment containing MB3 was cloned either upstream (0.7NP series) or downstream (NP0.7 series) of the natural *cdc25A* promoter as shown (b, bottom). CACGTG within MB3 in the 0.7-kb *SacI* fragment (MB3) in pGL3NP was changed to CAACTG an CACCTG by oligonucleotide-directed mutagenesis. Reporter plasmids were co-transfected into NIH3T3 cells together with pM21 (a wild-type *c-myc* expressing plasmid<sup>13</sup>) or deletion variants of *c-myc* in the same vector, obtained from T. deLange<sup>13</sup>. Deletions in the transactivation domain (Δ7–91), internal region (Δ145–262), helix-loop-helix (Δ371–411), and the leucine zipper (Δ414–433) were used. All transfections were supplemented with SV40 β-gal plasmid and carrier DNA to 20 μg. Luciferase activity was normalized against β-galactosidase activity of the same sample.

To see whether induction of *cdc25A* might be essential for Myc-driven apoptosis, we incubated Val5 cells with an antisense *cdc25A* oligonucleotide (Fig. 6 legend). Induction of Myc-OR by  $\beta$ -oestradiol and p53 by temperature shift normally results in apoptosis (ref. 37 and Fig. 6A). Apoptosis was inhibited by treatment with the antisense oligonucleotide, but not with a control scrambled one (Fig. 6A). To determine whether the antisense oligonucleotide had prevented Myc-driven induction of the *cdc25A* protein, we incubated Val5 cells with antisense oligonucleotide (Fig. 6 legend) and monitored the Cdc25A level

by immunoblotting. Induction of Cdc25A following  $\beta$ -oestradiol treatment was inhibited by the antisense but not by the scrambled oligonucleotide (Fig. 6A).

To extend this observation, we prepared an ecotropic retrovirus that expressed a *cdc25A* genetic-suppressor element (GSE; ref. 42), corresponding to the antisense coding sequence of *cdc25A*, and introduced it into rat Myc-OR cells (Fig. 6B). Cells infected with pBABE-puro retrovirus were used as a control. The level of Cdc25A was reduced  $\sim 4$ -fold in a pool of  $2-4 \times 10^5$  *cdc25A* GSE transformants (data not shown). We reasoned that cells in

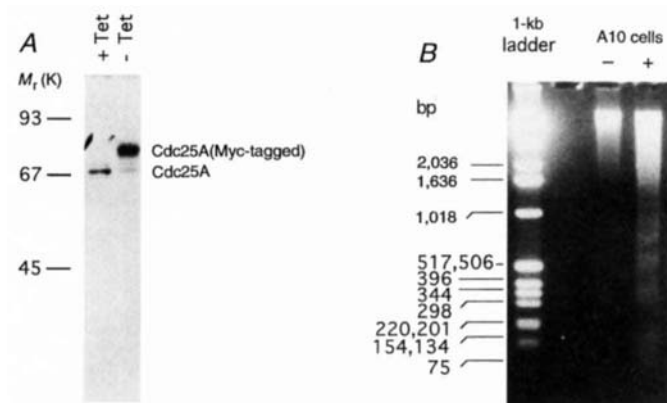
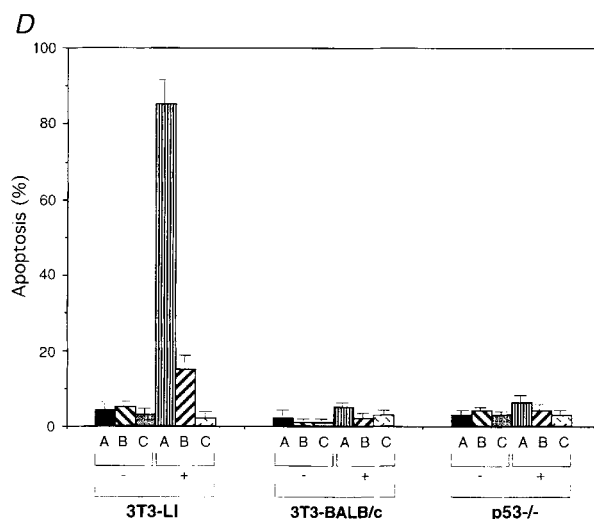
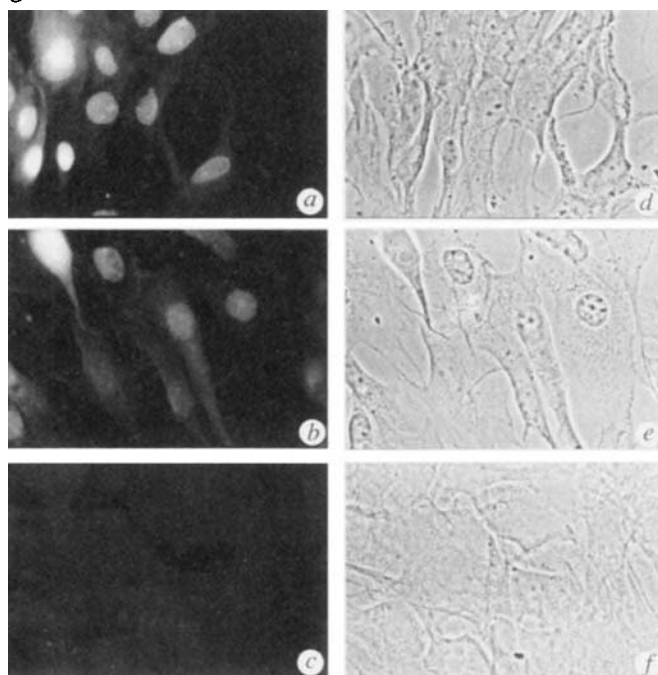


FIG. 5 Cdc25A induction of apoptosis. **B**, Western blot analysis of the tetracycline(tet)-regulated expression of *cdc25A* in serum-starved 3T3 LI/A10 cells. — and +, Presence or absence of tetracycline in the medium. **B**, DNA ladder in 3T3 LI/A10 cells after induction of *cdc25A*. — and +, Before and after induction of *cdc25A* by removal of tetracycline from the medium. **C**, TUNEL assay of 3T3 LI/A10 cells following induction of *cdc25A* for 18 (a, d) or 36 h (b, e); c, f, controls (no induction). a–c, TUNEL assay; d–f, phase contrast microscopy. Note the presence of the condensed chromatin granules in TUNEL-positive nuclei. **D**, induction of cell death following transient transfection of 3T3LI, 3T3 BALB/c or p53<sup>-/-</sup> MEF cells with *cdc25A*, *cdc25B* and *cdc25C* (A, B, C, respectively). + and –, Incubation in 0.1 and 10% FCS, respectively.

**METHODS.** An oligonucleotide, encoding the 9E10 antibody-reactive epitope (Myc-tag) was ligated to the *Nco*I-cut 4g4 plasmid containing the complete *cdc25A* open reading frame. This plasmid, expressing Myc-tagged Cdc25A (pcdc25AM) was digested with *Eco*RI and the resultant 2.4-kb fragment was transferred into pUHD10-3 (ref. 49) under the control of a tetracycline-repressible promoter (pcdc25AMtet). pcdc25AMtet, together with the plasmid carrying the tetracycline repressor/VP16 fusion protein<sup>49</sup> and containing a neomycin-resistance marker, was introduced into the 3T3 LI cell line (ATCC). Stable transfectants were selected with 400  $\mu$ g ml<sup>-1</sup> G418. Two colonies (out of 12 checked) were found to induce expression of *cdc25A* upon removal of tetracycline (A10 and A12 cell lines). Expression of the Myc-epitope-tagged Cdc25A was detected by reduced mobility of the protein recognized by anti-Cdc25A antibodies or by reaction with 9E10 antibodies (not shown). To study the effects of *cdc25* overexpression, cells were incubated for 48 h in DMEM containing 0.1% serum in the presence of 1  $\mu$ g ml<sup>-1</sup> tetracycline, followed by induction of *cdc25A* expression by changing to medium lacking tetracycline. Upon induction, cells were monitored and processed for TUNEL and light microscopy with a fluorescein-based ApopTag kit (Oncor). Cells were processed as recommended by the manufacturer, except that after fixation in 4% formalin, cells were permeabilized in 80% ethanol for 30–60 min at 4°C. Cells were photographed on a Zeiss Axiophote fluorescence microscope. For the experiments described in **D**, 3T3 LI, 3T3 BALB/c and p53<sup>-/-</sup> MEF cells were co-transfected with plasmids containing human *cdc25A*, *B* and *C* under the control of the cytomegalovirus(CMV) promoter and pCMV CD20 as described in ref. 35; 24 h after transfection, cell incubation was continued either in complete medium, supplemented with 10% FCS (indicated by –) or in growth medium with 0.1% serum (indicated by +). Apoptotic figures were scored 24 h later after microscopic investigation of the cultures based on CD20 staining, indicative of successful transfection (more than 2%; usually 8–10%), and visible morphological changes indicative of cell death (membrane blebbing, chromatin condensation, and so on).



**C**



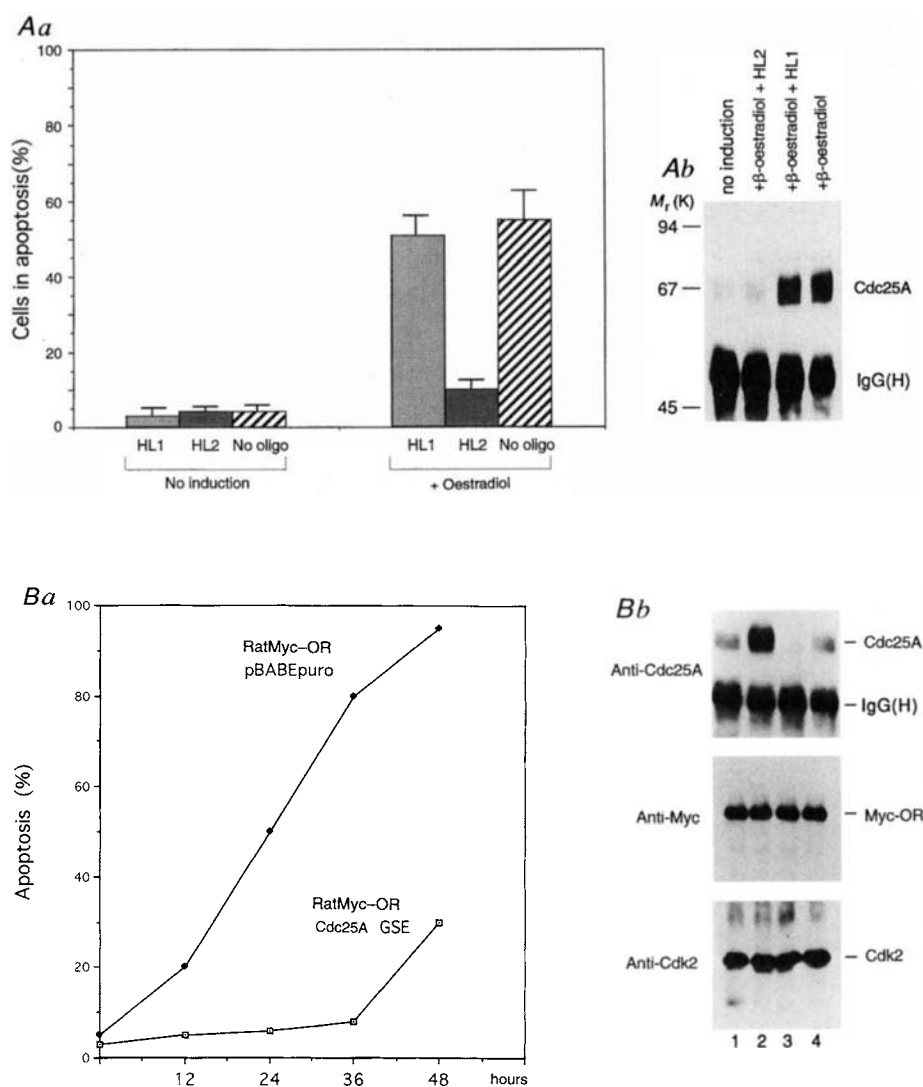
which *cdc25A* was effectively inhibited might be overgrown by cells expressing lower levels of the *cdc25A* GSE. We therefore subcloned *cdc25A* GSE and pBABEpuro-infected cell populations. Among the resultant *cdc25A* GSE colonies, about 70% corresponded to cells with a 'flat' appearance, like Rat1 cells expressing an inactive Myc mutant<sup>38,39</sup>. Ten per cent of the colonies were morphologically indistinguishable from Myc-OR cells, and 20% showed an intermediate phenotype. Essentially all of the control clones were indistinguishable from Myc-OR cells. The initial pools of infected cells, three randomly picked *cdc25A* GSE clones showing the 'flat cell' phenotype, and three vector-control colonies were further tested for Myc-induced apoptosis 24 h after Myc activation. Cell death was reduced by ~2–2.5-fold in the pool of *cdc25A* GSE-expressing clones compared with the pool of cells infected with the control virus. Individual GSE-

expressing clones displayed 5, 8 and 20% apoptotic cells versus 70, 80 and 95% in clones derived from the vector control. Inhibition of apoptosis correlated with a significant reduction in Cdc25A levels (Fig. 6Bb). There was no change in the levels of Myc-OR and Cdk2 proteins upon GSE expression (Fig. 6Bb). The *cdc25A* GSE protected cells from Myc-induced cell death for at least 48 h after Myc induction; however, we did observe some apoptosis in GSE-expressing cells at this point (Fig. 6Aa). This might indicate either that the *cdc25A* GSE did not completely protect against *cdc25A* induction by Myc, or that other Myc targets might compensate for the absence of *cdc25A*.

## Discussion

The *c-myc* proto-oncogene is a key regulator of cell growth and differentiation, but so far only the prothymosine and ornithine

FIG. 6 *cdc25A* is required for Myc-induced cell death. **A, a**, Inhibition of Myc-induced apoptosis in Val5 cells by an antisense oligonucleotide to *cdc25A*. HL1 corresponds to scrambled control oligonucleotide and HL2 to an antisense oligonucleotide to *cdc25A*; **b**, prevention of Cdc25A protein induction by  $\beta$ -oestradiol in Val5 cells, using Cdc25A antisense oligonucleotide, as indicated by western blot with anti-Cdc25A antibodies. **B, a**, Effect of *cdc25A* genetic suppressor element on apoptosis in RatMyc-OR cells. The time in hours after treatment with  $\beta$ -oestradiol and 0.1% serum is indicated. Squares, *cdc25A* GSA; diamonds, pBABEpuro control. **b**, Immunoprecipitation and western blotting to show inhibition of oestradiol-induced Cdc25A induction by *cdc25A* GSE. Lanes 1, 2, RatMyc-OR cells infected with control pBABEpuro retrovirus; 3, 4, RatMyc-OR cells infected with *cdc25A* GSE expressing retrovirus. Lanes 1, 3, cells in normal growth medium (10% serum); 2, 4, cells incubated for 24 h in 0.1% serum supplemented with 1  $\mu$ M  $\beta$ -oestradiol. **METHODS.** Val5 cells were grown in DMEM (without phenol red) in 10% fetal calf serum at 39 °C, shifted to 0.1% serum content at 32 °C for 24 h, then incubated for another 24 h with 200 nmol of a *cdc25A* antisense 17-mer S-oligonucleotide (obtained from Oligo Etc.), which spans the initiating methionine (CGGGCCAGTTCCATGG) of both human and mouse *cdc25A*, or with scrambled oligonucleotide (GGTACCTTGACCCGGGC) as a control. Incubations with oligonucleotides were done in the presence of an equimolar concentration of lipofectamin (Gibco BRL) for the first 24 h, followed by continuous incubation with 200 nmol oligonucleotide throughout the experiment. 48 h after shifting to 0.1% serum, the Myc-OR fusion protein was induced by addition of  $\beta$ -oestradiol, cell incubation was continued for 24 h, at which point cells were photographed and apoptosis was scored by TUNEL (Fig. 5 legend). Alternatively, cell extracts<sup>48</sup> were analysed by immunoprecipitation followed by western blot (Fig. 1 legend). For **B**, we prepared recombinant retrovirus by subcloning human *cdc25A* cDNA, including the complete open reading frame, as a 2.4-kb EcoRI fragment in the antisense orientation into pBABEpuro<sup>50</sup> (25AGSE). Recombinant ecotropic retrovirus was obtained after transfection into PhoenixE cells, provided by G. Nolan. RatMyc-OR cells<sup>39</sup> were infected with recombinant 25AGSE or with a control retrovirus and after 2 d were



selected with 2  $\mu$ g ml<sup>-1</sup> puromycin for 4 days. Pools of at least 2–4  $\times 10^5$  infected cells were obtained. These pools were single-cell subcloned by serial dilution, and resultant colonies were assayed for apoptosis by shifting cells to growth medium with 0.1% serum and 1  $\mu$ M  $\beta$ -oestradiol, or further expanded and subjected to either apoptosis or immunoprecipitation, followed by western blot analysis with anti-Cdc25A, anti-Myc or anti-Cdk2 antibodies as for Fig. 1.



decarboxylase genes have been described as direct and potentially relevant transcriptional targets<sup>28</sup>. Regulation of prothymosine by *c-myc* is cell-type-specific<sup>40</sup>. Ornithine decarboxylase transforms 3T3 cells *in vitro*<sup>43</sup>. However, small molecule inhibitors of this enzyme only marginally affect Myc-dependent apoptosis<sup>44</sup>. Therefore, neither ornithine decarboxylase nor prothymosine are likely to be the sole relevant targets of Myc<sup>28,40,44</sup>.

The following considerations lead us to suggest that *cdc25A* is one physiologically relevant Myc target. First, *cdc25A* mRNA and protein abundance are raised following activation of Myc. The protein synthesis inhibitor cycloheximide did not inhibit Myc-dependent activation of *cdc25A* transcription, suggesting that the effect of Myc on *cdc25A* transcription is direct. An increase in Cdc25A levels was not observed when a deletion mutant of Myc, lacking oncogenic and apoptotic activity<sup>12,38</sup> was used instead of the wild-type gene, thus correlating oncogenic properties of Myc with its ability to induce *cdc25A*. Second, the *cdc25A* gene contains three Myc/Max binding sites within the first two introns. These can direct Myc-dependent transcription from a heterologous promoter. Interestingly, Myc-binding sites are found downstream of the transcription initiation site in all known Myc transcriptional targets, including ornithine decarboxylase<sup>28,29,40</sup>. Finally, in the absence of adequate levels of growth factors, Cdc25A and Myc share the ability to induce p53-dependent apoptosis. Myc-driven apoptosis was inhibited by *cdc25A* antisense oligonucleotides and by a retroviral construct expressing a *cdc25A* genetic suppressor element<sup>42</sup>. This suggests that *cdc25A* expression might be essential for Myc-dependent apoptosis in some cells. We also observed that *cdc25B* is induced by Myc-OR, although at a later time and to a lesser extent than *cdc25A* (Fig. 1, and our unpublished observations). A single DNA fragment carrying a Myc/Max binding site was found in the *cdc25B* gene (result not shown). Thus, it is possible that *cdc25A* and *cdc25B*

might act cooperatively in Myc-driven cell-cycle activation and/or apoptosis.

Our conclusions are also supported by much circumstantial evidence that points to a potential role for Cdc25 as a mediator of Myc function. Myc stimulates the activity of cyclin-E- and cyclin-D1-dependent kinases (Cdk2 and Cdk4, respectively), without altering the levels of Cdk2, Cdk4, cyclin E or cyclin D1 (ref. 31). Cdc25 is a well established activator of cyclin-dependent kinases. Expression of *cdc25A*, which is essential for transition from G1 to S phase<sup>33,45</sup>, is growth-factor-responsive, peaking as early as 3 hours after serum stimulation of quiescent fibroblasts<sup>33</sup>. This coincides temporally with *c-myc* induction in the same cells<sup>46</sup>, and thereafter the levels of both *c-Myc*<sup>46</sup> and Cdc25A<sup>45</sup> are relatively constant during the cell cycle. The physiological substrates of Cdc25A and B have yet to be confirmed, but Cdk4 becomes tyrosine-phosphorylated after ultraviolet irradiation and Cdc25A can reverse this process<sup>47</sup>. Although the role of Cdk4 tyrosine-phosphorylation in non-irradiated cells is unclear, Cdk2 is regulated by tyrosine-phosphorylation in undamaged cells<sup>45</sup>. A key substrate of both Cdk4 and Cdk2 kinases is the retinoblastoma protein (Rb), and induction of Myc leads to Rb phosphorylation and inactivation<sup>30</sup>, presumably by the activated CDKs. Finally, *cdc25A* and *cdc25B* are able to cooperate with Ha-ras in oncogenic transformation of normal rodent fibroblasts<sup>35</sup>, a well-known property of *c-myc*.

Although it is plausible that *cdc25A* is causing cell-cycle progression by activation of Cdk2 (ref. 45) and possibly Cdk4 (ref. 47), the function of *cdc25A* in the induction of apoptosis is less clear. Regardless of the precise mechanism by which *cdc25* triggers apoptosis, our results point to an essential role for the cell-cycle phosphatases as targets of *c-myc* in normal growth control, oncogenesis and apoptosis. □

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# Earthquake-like behaviour of soft $\gamma$ -ray repeaters

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NEUTRON stars are believed to have solid crusts, which may exhibit tectonic activity analogous to earthquakes on Earth. These 'starquakes' have been invoked to explain several phenomena associated with neutron stars, such as the flashes of low-energy  $\gamma$ -rays from soft  $\gamma$ -ray repeaters<sup>1,2</sup> (SGRs), and abrupt changes ('glitches') observed in the spin periods of some pulsars<sup>3,4</sup>. Here we show that SGR events and earthquakes share four distinctive statistical properties: power-law energy distributions, log-symmetric waiting time distributions, strong positive correlations between waiting times of successive events, and weak or no correlations between intensities and waiting times. These statistical similarities, together with the fact that the crustal energy liberated by starquakes is sufficient in principle to fuel the soft  $\gamma$ -ray flashes, suggest that SGRs are indeed powered by starquakes.

An earthquake fault region can build up strain energy as high as  $\sim 10^{26}$  erg before producing a magnitude  $M \approx 9$  earthquake. Neutron stars, whose crusts have average densities  $\geq 10^{14}$  that of the Earth, can undergo global deformations (such as the twisting of one hemisphere relative to the other) which develop strain energies of  $\sim 10^{44}$ – $10^{46}$  erg (corresponding to  $M \approx 22$ ) before cracking occurs<sup>5,6</sup>. Starquakes of this size are large enough to power SGR events. The  $\sim 118$  events of SGR1806–20 each produced less than  $10^{42}$  erg in  $\gamma$ -rays<sup>7–13</sup>. The most energetic recorded SGR outburst, on 5 March 1979 from SGR0526–66, radiated  $\sim 4 \times 10^{44}$  erg; the other 15 events from SGR0526–66 were comparable in energy to those from SGR1806–20<sup>14,15</sup>. The frequent starquakes needed to produce SGR events require an energy source that can maintain a high level of strain in the crust. A possible candidate for this source is an evolving, super-strong stellar magnetic field of flux density  $\sim 10^{15}$  G (refs 16–18).

To find evidence of a starquake origin of the SGR events, we searched for similarities between earthquakes and SGR events. In

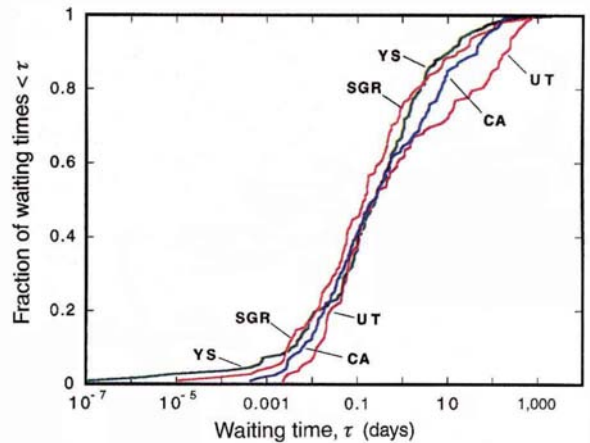


FIG. 2 The cumulative waiting-time (recurrence interval distributions for the events used in Fig. 1 with the same colour coding. The ordinate gives the fraction of events that have waiting times less than a value  $\tau$ .

particular, we compared the statistical properties of the distributions of the size and recurrence intervals of earthquakes and SGR events. For characterizing the statistics of SGRs we used the 111 events of SGR1806–20 observed during the 1979–84 period of continuous coverage with the ICE satellite<sup>7</sup>. For a representation of earthquake statistics, we examined three well studied seismically active regions: a tectonically active region in southern California, a volcanic region in Yellowstone National Park, and a mixed tectonic/volcanic region in Utah.

Figure 1 shows the cumulative energy distributions of events. The occurrence rate  $N$  of earthquakes with energies between  $E$  and  $E + dE$  obeys the well known 'Gutenberg–Richter' power law<sup>19</sup>:

$$\frac{dN}{dE} \propto E^{-\gamma}, \quad E < E_{\max} \quad (1)$$

with an index  $\gamma \approx 1.6$  with variations of  $\pm 0.2$  for different active regions. As the observed indices  $\gamma$  are less than 2, the rate of the energy release ( $\propto \int E(dN/dE)dE \propto E_{\max}^{2-\gamma}$ ) is dominated by the largest earthquakes; the cutoff energy  $E_{\max}$  has to be finite. The curves in Fig. 1 represent cumulative Gutenberg–Richter relations with indices 1.63, 1.70 and 1.61 for the California, Yellowstone and Utah regions, respectively. In none of these data sets

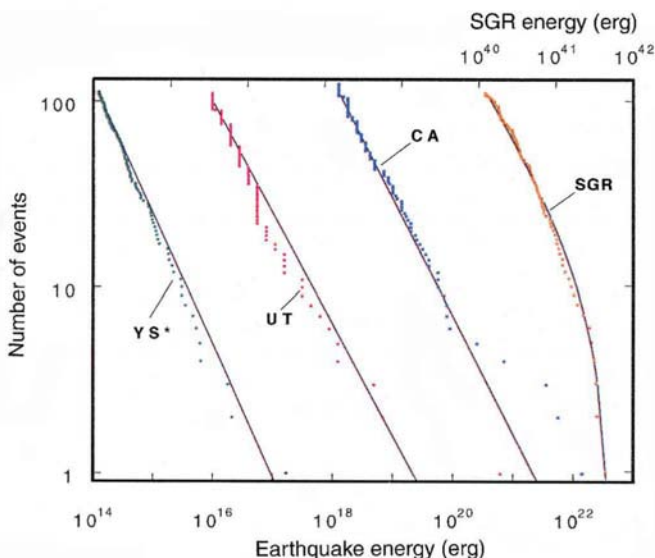
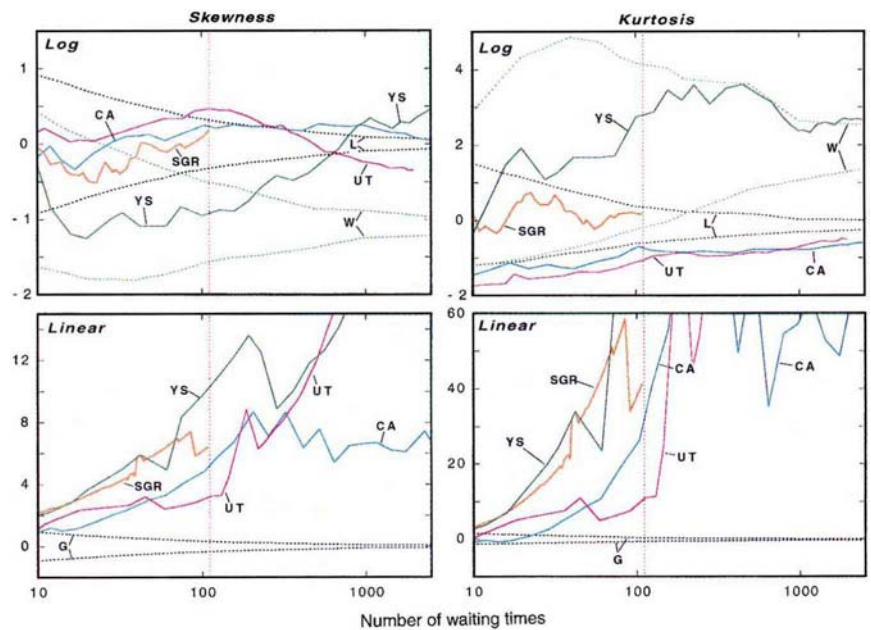


FIG. 1 Cumulative size distributions of the  $\sim 111$  strongest earthquakes from seismically active regions in California (blue; CA)<sup>29</sup>, Yellowstone National Park (green; YS\*)<sup>30</sup> and Utah (red; UT)<sup>31</sup> and 111 events from SGR1806–20 (orange; SGR)<sup>7</sup>. The ordinate is the number of events above energy  $E$ . The earthquake energies (in ergs) were obtained from reported magnitudes  $M$  using  $\log_{10}(E) = 11.8 + 1.5M$ . The Yellowstone data are shifted to the left by a factor of 10 in  $E$  for visual clarity (indicated by an asterisk). The energy scale for the SGR events is on the top border of the figure. The full earthquake data sets are as follows: California, 2,646 events between 1989 and 1994 within latitudes 33–35° and longitudes 115–121°; Yellowstone, 3,487 events between 1972 and 1991 within latitudes 44.60–44.68° and longitudes 110.94–111.06°; Utah, 1,972 events between 1962 and 1994 within latitudes 41.79–42.14° and longitudes 112.45–112.74°. The SGR1806–20 data consists of 111 events of detected with the ICE satellite between 1970 and 1984 (ref. 7). The theoretical curves give the cumulative distributions for Gutenberg–Richter power laws; that is,  $N(>E) \propto E^{1-\gamma} - E_{\max}^{1-\gamma}$ .

FIG. 3 Skewness and kurtosis measures of the waiting-time distributions for the earthquake and SGR1806 – 20 data sets colour coded as in Figs 1 and 2. The rightmost point for each distribution represents the entire data set, and the other points characterize sub-sets obtained by raising the energy thresholds. The upper-left panel gives the log- $\tau$  skewness,  $\langle(\log \tau - \langle \log \tau \rangle)^3\rangle/\sigma^3$  with  $\sigma^2 \equiv \langle(\log \tau - \langle \log \tau \rangle)^2\rangle$ , where  $\langle \rangle$  denotes an average over the data set. This quantity is a measure of the symmetry of the differential waiting time distribution when the independent variable is  $\log \tau$ . The upper-right panel gives the log- $\tau$  kurtosis,  $\langle(\log \tau - \langle \log \tau \rangle)^4\rangle/\sigma^4 - 3$  (the kurtosis is positive if the wings of the distribution are wider than those for a lognormal distribution and negative if they are narrower). The black dashed lines labelled L give the 95% confidence bounds for lognormal distributions ( $dN/d\log \tau \propto \exp\{-(\log \tau - \langle \log \tau \rangle)^2/\sigma_\tau^2\}$ ), and the green dashed lines labelled W give the 95% confidence bounds for Weibull distributions ( $dN/d\tau \propto \tau^{i-1}$ ). The lower panels give the linear- $\tau$  skewness and kurtosis, obtained by replacing  $\log \tau$  with  $\tau$  in the above definitions. The black dashed lines labelled G give the 95% confidence bounds for gaussian distributions ( $dN/d\tau \propto \exp\{-(\tau - \langle \tau \rangle)^2/\sigma_\tau^2\}$ ).



was the cutoff at  $E_{\max}$  apparent. Additionally, there was no significant difference between the power law indices of tectonic and volcanic regions.

The events of SGR1806 – 20 are similarly matched with a Gutenberg–Richter cumulative distribution with  $\gamma = 1.66$ , a value that falls within the observed range for earthquakes. The noticeable difference between the SGR1806 – 20 and earthquake distributions is that the former's high-energy cutoff is detectable at  $E_{\max} \approx 5 \times 10^{41}$  erg.

The waiting time is the interval between successive events in a data set. Figure 2 shows the cumulative waiting-time distributions  $W(\tau)$ ; that is, the fraction of all the event pairs that have waiting times less than  $\tau$ . This figure suggests that the SGR events and the earthquakes have similar waiting-time distributions. Note the large widths of the distributions and the substantial tails extending at least two orders of magnitude above and below the median. To compare these distributions quantitatively, Fig. 3 displays their skewness and kurtosis as a function of the number of events above threshold. These statistics show that the shape of the waiting-time distribution for SGR1806 – 20 falls in the range delineated by the observed earthquakes. There is no obvious difference between the waiting-time distributions for the tectonically active, volcanic or mixed regions.

It has been suggested that the waiting-time distributions of earthquakes can be described by lognormal<sup>20</sup>, Weibull<sup>21</sup> or gaussian<sup>22</sup> distributions and that the waiting-time distributions of SGR events can be fitted by lognormal distributions<sup>23</sup>. To test these ideas we used Monte Carlo simulations to derive the 95% confidence intervals for finite samples randomly drawn from gaussian, lognormal and Weibull distributions. The dashed lines labelled G in the lower panels of Fig. 3 give the confidence intervals for the gaussian distribution, and those labelled L and W in the upper panels give the confidence intervals for lognormal and Weibull distributions, respectively. The SGR waiting times are consistent with a lognormal distribution, but none of the earthquake data sets are statistically consistent with either lognormal or Weibull and they are grossly different from gaussian.

We have found that there are strong correlations between the magnitudes of the waiting times for both active earthquake regions and for SGR1806 – 20; that is, if the logarithm of the interval between two events is short (or long), then the logarithms of the intervals between successive events also tend to be short (or long). This property is illustrated in Fig. 4 which shows the log- $\tau$

waiting-time autocorrelation function  $g(k)$  for intervals separated by  $k$  events. The correlation of SGR1806 – 20 events is in the same general range as that found for the earthquake events; all of the earthquake regions and SGR1806 – 20 show strong correlations for  $k \leq 2$ .

We find little or no correlations between waiting times and energies for SGR1806 – 20 and for the earthquake regions; this confirms the findings of other workers<sup>7,24</sup>. This lack of correlation distinguishes SGR events from cosmic X-ray bursts that have energies nearly proportional to the preceding waiting times<sup>25–27</sup>. In this latter phenomenon the reservoir of stored energy (believed to be accreted matter that undergoes nuclear burning) is largely depleted during an outburst and must be replenished before the next event. In earthquakes, and presumably starquakes, the reservoir of strain energy is hardly depleted by the most common, small events. There are long-term trends showing that the rate of energy released in an earthquake region averaged over years is nearly constant<sup>28</sup>, but this trend is not apparent in the limited data available for SGR events.

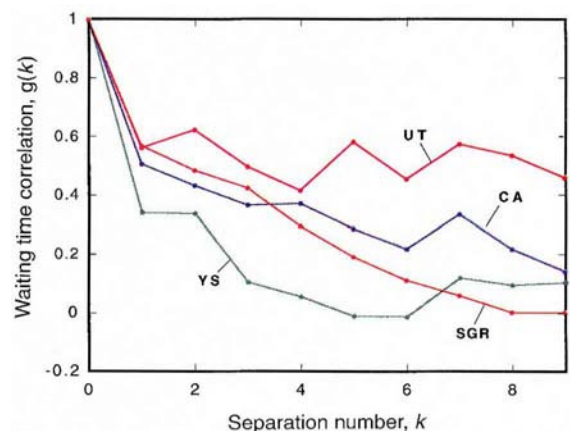


FIG. 4 The log- $\tau$  waiting-time autocorrelation function:  $g(k) \equiv G(k)/G(0)$ , where  $G(k) \equiv \langle(\log \tau_{i+k} - \langle \log \tau \rangle)(\log \tau_i - \langle \log \tau \rangle)\rangle$ . Here  $\langle \rangle$  denotes an average over all possible values of  $i$  while  $k$  is held fixed.



The statistical similarities between earthquakes and SGR events argue for physically similar origins. This conclusion is strengthened by the physical argument that the build-up and release of strain in the neutron-star crust is one of the few ways that the energy requirements for SGR events can be satisfied. One should be cautious, however, in fully accepting that SGR events derive from starquakes. One significant feature of earthquake statistics, namely a flood of small aftershocks following an especially large event, is not obvious in SGR statistics (though the largest event from SGR0526 – 66 was closely followed by two smaller events). We note that although earthquakes and SGR events do share the four statistical features discussed here, we have not shown that these features are unique to earthquakes and starquakes; conceivably, other nonlinear dynamical phenomena may exhibit these features. □

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## Very low viscosity at the solid–liquid interface induced by adsorbed C<sub>60</sub> monolayers

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MOLECULES of C<sub>60</sub> (refs 1–4) are near-perfect spheres, and in the crystalline solid each molecule rotates rapidly while remaining at its lattice position. Speculation that this property may be conducive to good lubrication has not been borne out by experiments, at least for C<sub>60</sub> on its own<sup>5–8</sup>. Here we report measurements of the intermolecular interactions of C<sub>60</sub> adsorbed as monolayers on surfaces immersed in a liquid. We find that the adsorbed layers impart a surprising effect on the flow properties of the liquid at the surface. The measured short-range oscillatory force indicates weak adsorption of one to two layers of C<sub>60</sub> on each surface. In the presence of adsorbed layers, fluid flow between two surfaces exhibits full-slip (zero drag) boundary conditions, giving rise to flow behaviour that is totally different from conventional fluid flow through narrow pores. This suggests that C<sub>60</sub> might be effectively used as an additive to conventional lubricant fluids.

We used the surface-force apparatus<sup>9,10</sup> (SFA) to measure the interactions between molecularly smooth and optically transparent sheets of mica coated with C<sub>60</sub> layers, both in air and across solutions of toluene containing dissolved C<sub>60</sub>. We first describe the forces measured in solutions of toluene containing C<sub>60</sub>. In pure dry toluene (in the absence of C<sub>60</sub>), the 'control' force was found to be a decaying oscillatory function of distance with an average periodicity of 5.5 Å—the mean diameter of a toluene molecule. This oscillatory force profile is in agreement with both previous experiments and theoretical expectations<sup>11,12</sup>, and is readily explained by the ordering of toluene molecules into quasi-layers between the flat mica surfaces.

In the next set of experiments, the force profile in pure toluene

was first measured and ascertained to be oscillatory—an indication that the solvent was pure and dry. To this, small quantities of the stock solution (1 mg C<sub>60</sub> per ml toluene) were then added to bring the final concentration in the SFA chamber to the desired value. Figure 1 shows the measured force across a solution of C<sub>60</sub> (0.095 mg C<sub>60</sub> per ml solution). The profile is again oscillatory, but

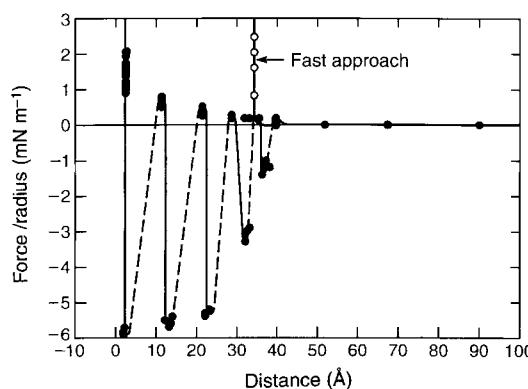


FIG. 1 Measured forces  $F$  as a function of surface separation  $D$  between two curved mica surfaces each of radius  $R \approx 1\text{--}2\text{ cm}$  in toluene containing 0.095 mg per ml dissolved C<sub>60</sub>. Zero distance ( $D = 0$ ) is based on mica–mica adhesive contact in dry nitrogen atmosphere. An oscillatory force profile is measured when the surfaces are moved slowly (filled circles)—the solid line being the experimentally measured profile (some points lying between the extrema have been omitted for clarity). The dashed regions are experimentally inaccessible regions. A monotonic repulsion is measured when the surfaces are brought together quickly (empty circles).

METHODS. Solid C<sub>60</sub>, 99.5% pure, was obtained from Bucky, Houston, Texas, USA. Stock C<sub>60</sub> solutions were made at a C<sub>60</sub> concentration of 1 mg per ml using Optima grade toluene (Fischer) which was stored with molecular sieves and distilled under nitrogen before being used. The water content of the solutions was 0.05%. All solutions were filtered using a pre-cleaned 0.2-μm PTFE filter while introducing the solution into the SFA. Normal forces were measured from the deflection of a horizontal spring supporting one of the two surfaces<sup>10</sup> and distances were measured using an optical technique (based on fringes of equal chromatic order (FECO) interferometry) that allowed measurement of surface separations and spring deflections with a resolution of  $\sim 1\text{ Å}$  (ref. 28).

the periodicity is now  $10.5 \pm 0.5 \text{ \AA}$ , the diameter of the  $C_{60}$  molecule, and indicative of the adsorption of one or two monomolecular layers of  $C_{60}$  on each surface.

The oscillatory force profile is not unexpected, but unusual and previously unrecorded dynamic effects were also observed. Two commonly occurring forms of the measured force profiles are shown in Fig. 1. If the surfaces were made to approach rapidly (faster than  $50 \text{ \AA s}^{-1}$ ), the force profile showed an incompressible 'hard-wall' repulsion at a distance of  $34 \text{ \AA}$  (open circles). This repulsion is attributed to the steric repulsion of four layers (two per surface) of  $C_{60}$  molecules kinetically trapped between the surfaces. If the speed of approach of the surfaces was reduced, or if pauses occurred during the approach, the oscillatory force profile shown in Fig. 1 (filled circles) was obtained. These rate-dependent effects were absent in pure toluene, or indeed in other liquids composed of relatively small molecules, either pure or in solution. Some previous studies have also indicated some variation in the heights and locations of oscillatory and steric repulsions as a function of speed<sup>13</sup>, but never as dramatic an effect as was seen in this system. Figure 1 also shows that the surfaces could be brought into molecular contact (at surface separation  $D = 0$ ) with relative ease, which is also unusual<sup>14–16</sup>. In all, the normal-force measurements indicated that the adsorbed layers possess unusually high fluidity and are easily disrupted.

A related and much more intriguing feature of this system is the viscous response of the fluid near the surfaces. Viscous forces, the exact locations of slipping planes, and the viscosities of liquids in ultra-thin films and near surfaces may be measured to high accuracy using the SFA<sup>17,18</sup> (Fig. 2). Previous results on various liquids in ultra-thin films between various surfaces have indicated that simple liquids and liquid mixtures generally remain newtonian and 'bulk-like' in films as thin as  $25 \text{ \AA}$ , and that the equations of viscous flow obey the expected non-slip boundary condition (equation (1) in Fig. 2 legend) at the solid–liquid interface<sup>17–21</sup>.

For pure toluene, our experimental results fit well with the analysis for a fully no-slip boundary condition (Fig. 2, line through filled squares, equation (1)) giving a bulk viscosity of  $0.53 \pm 0.04 \text{ cP}$  and a shear plane, as deduced from the  $D$ -axis intercept, of  $33 \pm 8 \text{ \AA}$ . This is exactly the same result as found in a previous experiment<sup>22</sup> (although the point must be made that in the prior experiment the toluene was 'wet' and exhibited a non-oscillatory, purely attractive force). This confirmation of the viscosity results also shows that the short-range force profile does not affect the liquid viscosity beyond the range of the structural oscillatory force (compare Fig. 1).

When the same type of analysis was performed on the system with an adsorbed layer of  $C_{60}$  molecules, the results were completely different for what should be essentially the same bulk liquid viscosity. The only reasonable explanation for the experimental results, both qualitatively and quantitatively, is that the system is exhibiting a full-slip boundary conditions at both surfaces, as described by equation (2) (Fig. 2 legend). This fit (Fig. 2, line through filled circles) gives a bulk viscosity of  $0.55 \pm 0.06 \text{ cP}$  which agrees with the literature value for toluene<sup>23</sup> of  $0.57 \text{ cP}$ . It follows that  $C_{60}$  is involved in a very unusual molecular interaction that results in the boundary exhibiting full slip (as does a vapour–liquid surface in, for example, a free liquid film in air). An explanation for this result is that the adsorbed layer of molecules interact very weakly with the surface, as already evidenced by the weak binding and high sensitivity to approach rate of the force profiles, and that they may also be rotating rapidly, as in the solid, producing an effective boundary of near-zero drag on the adjacent liquid.

Friction forces were also measured with an attachment to the SFA that measured lateral forces with strain gauges attached to a vertical friction-force measuring spring<sup>24</sup>. We studied the friction of  $500\text{-\AA}$ -thick solid layers of  $C_{60}$ , produced by the sublimation of  $C_{60}$  under vacuum on mica, sliding in air<sup>25</sup>. These experiments were carried out to confirm that the solid surface of  $C_{60}$  alone has

no obvious beneficial tribological properties<sup>5,6,26</sup>. The results (not shown) yielded a 'normal' kinetic friction coefficient of  $\mu = 0.15$ , independent of the sliding velocity, which agrees with previous results obtained on macroscopic systems in which solid  $C_{60}$  was used as a surface coating layer<sup>27</sup>.

The results reported here provide evidence for highly unusual behaviour of  $C_{60}$ , and perhaps of other large or similarly shaped molecules, adsorbed on solid surfaces from solution, giving rise to very effective drag reduction or lubricity on fairly thick liquid films (Fig. 2) yet due to a changed boundary condition for slip occurring entirely within one or two molecular layers at the surface. The results also indicate that  $C_{60}$ , although not a good lubricant, shows great promise as an additive. □

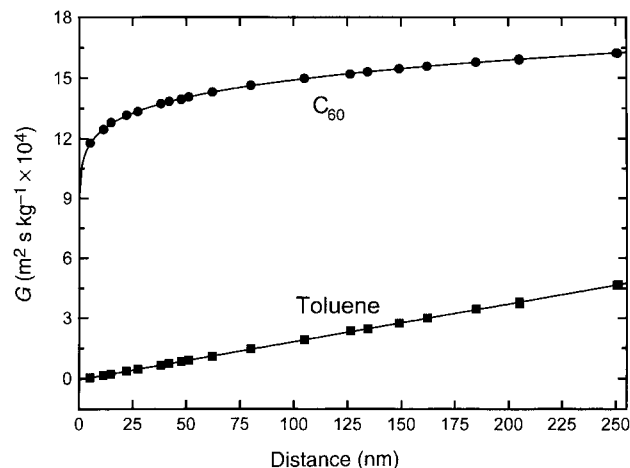


FIG. 2 Viscosity measurements for the same system as in Fig. 1 fitted to the following equation where  $G$  is defined by equations (1) or (2) below depending on the slip boundary condition (note that larger values of  $G$  imply smaller effective viscosities using the same model):  $G^{-1} = (K/12\pi^2 R^2 v) [(A_0/A)^2 - 1]^{1/2}$ . Lower curve (pure toluene), best fit to  $G$ , using equation (1) below ( $G = D/\eta$ ), for the conventional case of no-slip at the solid–liquid interface. The best-fit line gives a bulk viscosity of  $\eta = 0.53 \pm 0.04 \text{ cP}$  for the intervening liquid, which compares well with the literature value<sup>23</sup> for bulk toluene of  $0.57 \text{ cP}$ . Upper curve ( $C_{60}$  solution in toluene), best fit to  $G$ , defined by equation (2) below ( $G = RC/\ln(D/R)\eta$ ), for the highly unusual case of full-slip at the solid–liquid interfaces. The best-fit line gives a bulk viscosity of  $\eta = 0.55 \pm 0.05 \text{ cP}$  for the intervening liquid. The parameter  $C$  has a value of  $825 \pm 20$ . For both the lower and upper curves, the errors in the measurements are about the size of the data points along the vertical  $G$ -axis, and significantly less along the distance axis.

METHODS. Liquid viscosity was measured in the SFA by vibrating the upper surface, which was mounted on a piezoelectric crystal, and monitoring the response of the other surface<sup>17,19</sup>. For the case of no-slip boundary condition at the mica–solution (solid–liquid) interface the viscous drag force is given by  $6\pi R^2 \eta v/D$ , from which the viscosity  $\eta$  is given by<sup>17</sup>

$$\eta = \frac{KD}{12\pi^2 R^2 v} \left[ \left( \frac{A_0}{A} \right)^2 - 1 \right]^{1/2} \quad \text{for } R \gg D \quad (1)$$

where  $K$  is the force-measuring spring constant,  $D$  the mean surface separation,  $v = dD/dt$  the relative velocity of the moving surfaces,  $\nu$  the frequency of vibration,  $R$  the hydrodynamic radius of curvature,  $A_0$  the amplitude of vibration of the upper surface (the drive), and  $A$  is the amplitude of vibration of the surface separation (the response). For the case of full-slip at the boundaries, the viscous drag force is given by<sup>29,30</sup>  $6\pi R \eta v [\frac{1}{2} \ln(R/D) + C]$  where  $C$  is a constant. The solution to the equation of motion for positive  $C$  and  $\eta$  contains two roots, one root gives  $C$ , the other gives the viscosity  $\eta$  according to

$$\eta = \frac{K}{12\pi^2 RC \nu \ln(R/D)} \left[ \left( \frac{A_0}{A} \right)^2 - 1 \right]^{1/2} \quad \text{for } R \gg D \quad (2)$$

The constant  $C$  is a pure scaling constant that depends on the surface geometry, which could be obtained if the exact flow profiles were known. In these experiments, the constant is used as a fitting parameter because the flow system is too complex to determine exactly.

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## Colorimetric chiral recognition by a molecular sensor

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ONE of the most pressing challenges in the design of molecular sensors<sup>1–7</sup> is to achieve visual discrimination between enantiomers. A simple monitoring system for distinguishing between the left- and right-handed forms of chiral drugs would be extremely useful in pharmacology<sup>8</sup>. A molecular sensor that achieves this would have to translate an enantioselective molecular recognition event into a discernible colour change. Here we describe a system of this sort, in which a proton transfer and conformational change in a chiral calixarene-based receptor brought about by recognition of a chiral substrate cause spectral shifts in two chromophores attached to the binding cavity of the calixarene. Because the spectral shift in one chromophore is greater for one enantiomer of the substrate than for the other, binding is accompanied by a colour change that can be observed visually.

As part of our ongoing program to develop improved optical receptors for biologically and/or chemically important cations and amines, we have synthesized chromogenic calixarenes<sup>9–13</sup>. Calixarenes, which are cyclic phenol-derived macrocycles<sup>14–22</sup>, are particularly attractive frameworks for the construction of optical sensing systems<sup>10,12,13</sup> because chromophores can be appended to the calixarene platform<sup>23–36</sup>. This has led us to consider the possibility that such systems could be useful in the construction of sensors that allow the visual detection of, and enantiometric distinction between, amines and amino acids. Towards this end,

TABLE 1 Association constants\* *K* of molecule **1** with several chiral amines

| Receptor  | Amine    | <i>K</i> /dm <sup>3</sup> mol <sup>−1</sup> |            |
|-----------|----------|---------------------------------------------|------------|
|           |          | (R)-isomer                                  | (S)-isomer |
| <b>1a</b> | <b>2</b> | 66 ± 8.8†                                   | ND‡        |
| <b>1b</b> | <b>2</b> | 17 ± 12                                     | ND‡        |
| <b>1a</b> | <b>3</b> | ND‡                                         | ND‡        |
| <b>1a</b> | <b>4</b> | 159 ± 16                                    | ND‡        |

\* Benesi–Hildebrand plot was used: the data were based on the changes of absorbance at ~540 nm.

† Further evidence for the formation of a 1:1 complex was given by fast-atom bombardment mass spectrometry (*m*-nitrobenzyl alcohol matrix), where Xe was used as the atom beam accelerated to 3.0 keV, as indicated by the value of 1,181 (**1** + (**R**)-**2**).

‡ Addition of excess amine results only in the appearance of the long-wavelength band due to the acid(indophenol-OH)-base (amine) reaction, and does not induce a shift of the short-wavelength band.

based in part on CPK molecular modelling, we designed and synthesized the chiral calix[4]crown **1** (Fig. 1). As detailed below, this system, which contains an optically active 1,1'-binaphthyl subunit and two indophenol chromophores, does in fact serve as a visually detectable sensor for certain stereogenic guests.

System **1a** and its congener were synthesized from optically pure (*S*)-1,1'-bi-2-naphthol via a set of straightforward steps involving etherification in the presence of a base, tosylation with tosyl chloride, a modified Williamson synthesis with calix[4]arene<sup>37</sup> in the presence of base, and a condensation with 4-amino-*m*-cresol under alkaline conditions in the presence of K<sub>3</sub>[Fe(CN)<sub>6</sub>]. Proton NMR analysis confirmed that both **1a** and **1b** contain 1,1'-binaphthyl- as well as the bis(indophenol)-derived calix[4]crown unit. It also showed that the system was in the cone conformation<sup>14</sup>. Altering the length of the ether spacers in **1a** allows the position of the binaphthyl unit to be closer to one of this

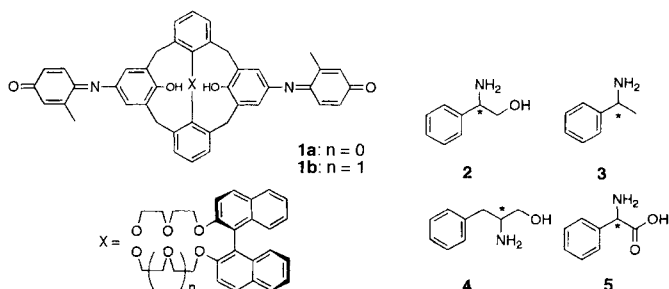


FIG. 1 Structures of dual optical sensory systems **1** and guest amines and amino acid derivatives.



indophenols than the other. Because of this, the two indophenol groups are distinct, as evidenced by the chemical shifts of two indophenol OH protons ( $\delta$  7.91 and  $\delta$  8.06 p.p.m.) in  $^1\text{H}$  NMR spectra. On binding of a chiral substrate to **1a**, each of the two chromophores will be affected to different extents, and we reasoned that this might translate into a powerful chromogenic response.

To test this hypothesis, compound **1a** was dissolved in ethanol to give a red solution having an absorption band at 515.5 nm ( $\epsilon_{\text{max}}$  14,500 dm<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup>). (*R*)-phenylglycinol, (*R*)-**2**, was then added as a putative response-inducing guest at 25 °C, resulting in an immediate colour change to blue-violet, reflected in the spectral shift (23 nm) in the band originally at 515.5 nm and the appearance of a new absorption band at ~650 nm (Fig. 2a). Subsequently, the absorbance peaks of the two new bands at 538 nm (short-wavelength band) and at 652.5 nm (long-

wavelength band), respectively, increased (isosbestic points at 416 and 505 nm). A Benesi-Hildebrand plot<sup>38</sup> for a mixture of **1a** and (*R*)-**2** in ethanol at 25 °C supported a 1:1 stoichiometry of the complex. The apparent association constant *K* was calculated as  $66 \pm 8.8$  dm<sup>3</sup> mol<sup>-1</sup>. Interestingly, when a similar experiment is carried out using the corresponding enantiomer, (*S*)-**2**, the solution remains red (Fig. 2b), with almost no detectable change in the spectrum (even in the presence of 10<sup>3</sup> equivalents of the isomer). Taken together, these results indicate that distinction between enantiomers can be successfully achieved by the naked eye (Fig. 2c).

In an effort to understand the details of the substrate-receptor interactions, congener **1b**, containing a large binaphthyl-derived crown ether unit, was studied. When this species was tested as a putative complexing agent for (*R*)-**2**, a *K* value ( $17 \pm 12$  dm<sup>3</sup> mol<sup>-1</sup>) was obtained that is about a quarter of that of **1a** + (*R*)-**2**

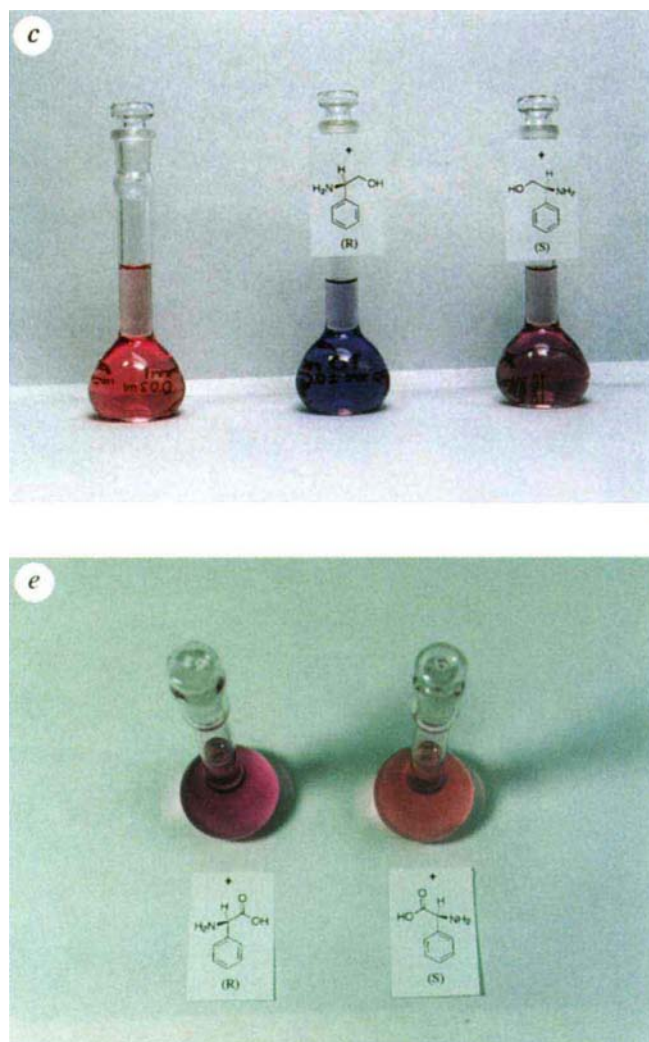
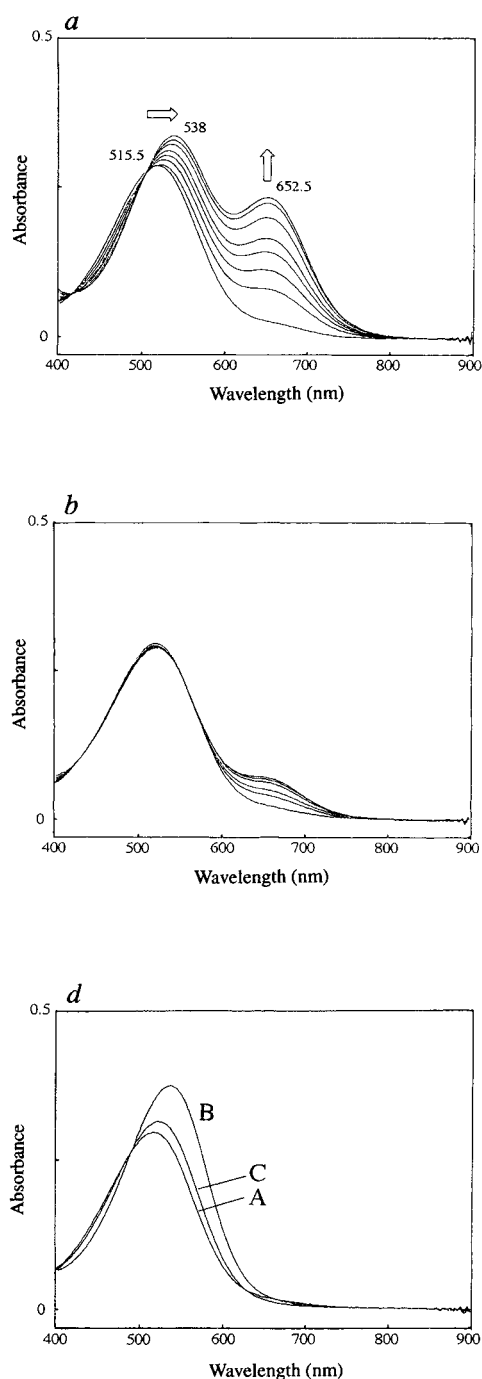


FIG. 2 a, Spectral change upon addition of (*R*)-**2** ( $0-2.0 \times 10^{-2}$  mol dm<sup>-3</sup> bottom to top) to an ethanol solution of **1a**: concentration of **1a**,  $2.0 \times 10^{-5}$  mol dm<sup>-3</sup>; 25 °C. b, Spectral change upon addition of (*S*)-**2** ( $0-2.0 \times 10^{-2}$  mol dm<sup>-3</sup> bottom to top) to an ethanol solution of **1a**:  $2.0 \times 10^{-5}$  mol dm<sup>-3</sup>; 25 °C. c, Visible difference between enantiomers of **2**: left, receptor **1a**; centre, after addition of (*R*)-**2**; right, after addition of (*S*)-**2**. d, Absorption spectra of **1a** after extraction with solid (phenylglycine **5**)–liquid(ethanol; 25 °C) two-phase solvent as **5** is insoluble in ethanol:  $[\mathbf{1a}] = 2.0 \times 10^{-5}$  mol dm<sup>-3</sup>; A, **1a**; B, **1a** + (*R*)-**5**; C, **1a** + (*S*)-**5**. e, Visible difference between the enantiomers of **5** after solid–liquid solvent extraction: left, **1a** + (*R*)-**5**; right, **1a** + (*S*)-**5**.

(Table 1). This indicates that the amine complexing process, in the case of **1a** and **1b**, take place in the cavity involving the ether oxygens of the macro-ring and phenolate of the indophenol. Furthermore, no selectivity for (*R*) or (*S*)-phenylethylamine **3** was obtained for **1a**, also supporting the argument that the alcohol OH group of the initial amine guest **2** participates in chiral recognition, probably as a result of hydrogen bonding involving the phenolate of the indophenol. Figure 3a suggests a possible structure for the complex of **1a** with a chiral amine. The amine-induced colour change observed with **1a** upon addition of (*R*)-**2** is ascribed to ionization of the phenol unit of the indophenol chromophore on the one hand (long-wavelength band) and a binaphthyl-induced hydrophobic effect on the other (short-wavelength band); in the latter instance, binding of the amine substrate is thought to alter the position of the binaphthyl unit in a way that is not possible in the case of the corresponding enantiomer (*S*)-**2**, which may then be strengthened by intramolecular hydrogen bonding between the indophenol OH and the adjacent phenolic oxygen in system **1a**, bringing about the spectral change of the short-wavelength band.

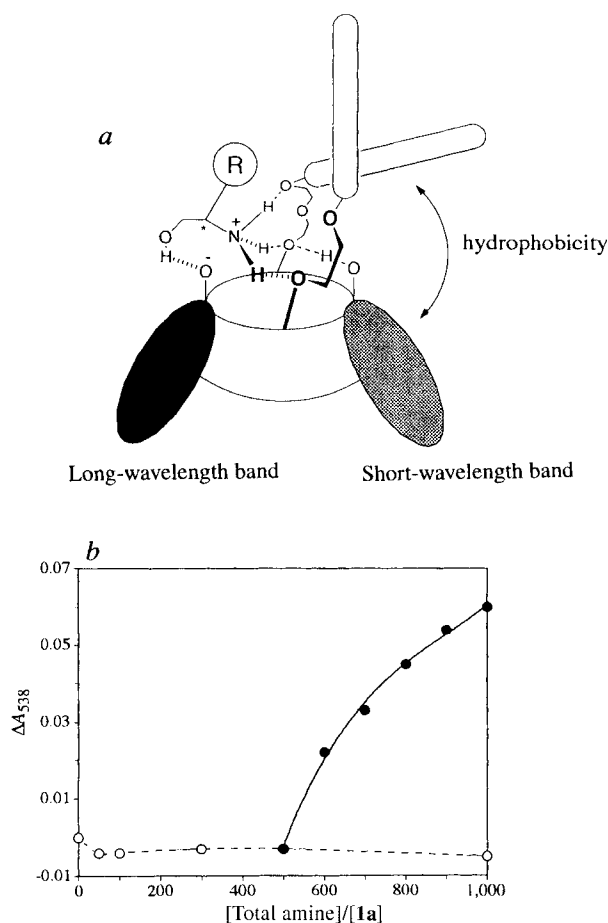


FIG. 3 a, Possible structure for the complex of **1a** with a chiral amine such as **2**. In order to display enantioselective binding to the chiral amine, system **1a** requires three recognition groups (ethyl oxygens, indophenol O<sup>-</sup> and a binaphthyl group); the ethyl oxygens and indophenol O<sup>-</sup> stabilize the host-guest complex and restrict the rotation of the guest along the C<sup>+</sup>-NH<sub>3</sub><sup>+</sup> axis via ion-dipole and hydrogen-bonding interactions. The third recognition group, the binaphthyl group, acts as a minor steric-repulsive site for the (*R*) isomer, and as a major steric-repulsive site for the (*S*) isomer. b, Competition between the enantiomers of **2** in an ethanol solution of **1a**; the figure shows the absorption intensity at 538 nm as a function of added equivalents of guest ([total amine]/[**1a**]) at 25 °C, where [**1a**] =  $2.0 \times 10^{-5}$  mol dm<sup>-3</sup>, open circles represent added (*S*)-**2**, and filled circles are added (*R*)-**2** in the presence of 0.01 mol dm<sup>-3</sup> (500 equivalents) of (*S*)-**2**.

Having defined the basic features of the binding process, we tested the potential application of **1a** as a colorimetric sensor. In a competition experiment, we recorded the changes in absorption intensity upon addition of increasing amounts of (*R*)-**2** to an ethanol solution of **1a** in the presence of  $1.0 \times 10^{-2}$  mol dm<sup>-3</sup> of (*S*)-**2**. As shown in Fig. 3b, even the addition of low concentrations of (*R*)-**2** under these conditions results in a marked increase in absorption intensity. Based on these findings, we are confident in predicting that receptor **1a** could be used to determine varying concentrations of (*R*)-**2** precisely, even in the presence of (*S*)-**2**.

Note that receptor **1a** is not limited in its utility to the simple chiral substrate **2**. Rather, it shows enantiomeric discrimination in the case of phenylalaninol salts such as **4** (Table 1), as well as certain amino-acid derivatives such as phenylglycine **5**. In the case of this latter, physiologically more interesting substrate, the absorption spectrum of **1a** was recorded after solid(**5**)-liquid ( $2.0 \times 10^{-5}$  mol of **1a** in ethanol at 25 °C) two-phase solvent extraction. Addition of (*R*)-**5** caused only a 20-nm shift in the short-wavelength band (but increased absorbance; compared with Fig. 2d), but a substantial shift was observed in the visible region (from 500 to 600 nm), a change reflecting the red-to-reddish-violet colour change under these conditions (Fig. 2e). On the other hand, when the (*S*)-**5** was added, there was virtually no discernible change in the absorption spectrum of **1a**.

We conclude that system **1a** not only represents a rationally designed colorimetric sensor for chiral substrate recognition, but also suggests a new general strategy for visual distinction between enantiomers, which should be applicable to a wide range of biologically relevant substrates. □

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# A self-replicating peptide

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**THE production of amino acids and their condensation to polypeptides under plausibly prebiotic conditions have long been known<sup>1,2</sup>. But despite the central importance of molecular self-replication in the origin of life, the feasibility of peptide self-replication has not been established experimentally<sup>3-6</sup>. Here we report an example of a self-replicating peptide. We show that a 32-residue  $\alpha$ -helical peptide based on the leucine-zipper domain of the yeast transcription factor GCN4 can act autocatalytically in templating its own synthesis by accelerating the thioester-promoted amide-bond condensation of 15- and 17-residue fragments in neutral, dilute aqueous solutions. The self-replication process displays parabolic growth pattern with the initial rates of product formation correlating with the square-root of initial template concentration.**

An autocatalytic process in which the reaction product serves as the specific catalyst for its own synthesis by recognizing and promoting coupling of reactant(s) is the most basic form of molecular self-replication (Fig. 1). A few examples of non-enzymatic template-directed autocatalytic systems have been described in which the primary molecular recognition event is based on nucleic-acid-like base-pairing interactions<sup>7-16</sup>. Unlike the nucleic acid bases, which provide a clear framework for establishing complementary molecular interactions, information transfer in polypeptides is inherently more complex and dependent on both the structure and the polypeptide sequence. The present design of the peptide replicator is based on one of the simplest forms of natural protein tertiary structure: namely the  $\alpha$ -helical coiled coils. These structures are distinguished by their amphiphilic primary sequence of heptad repeats (abcdefg)<sub>n</sub>. The first (a) and fourth (d) amino-acid residues make up the hydrophobic core which defines the complementary interhelical recognition surface. Recent studies have established that the identity of the amino acids in these positions and the specificity in the side-chain packing interactions in the hydrophobic core are important determinants of the structures and the aggregation state of coiled coils<sup>17,18</sup>. Conceptually, a given  $\alpha$ -helical subunit of a coiled-coil structure can be viewed as a template acting cooperatively to organize other participating peptide subunit(s). We considered that if a similar templating effect could also operate on shorter complementary peptide fragments, it may constitute the basis of an autocatalytic cycle for peptide self-replication.

The self-replication process described is based on a 32-residue peptide similar in sequence to the leucine-zipper homodimerization domain of the yeast transcription factor GCN4 (Fig. 2). The sequence differs from the natural sequence in six mutations out of the 32 residues, five of which are away from the recognition surface: two tyrosine residues were placed on the solvent-exposed surface to provide spectroscopic handles, and alanine and cysteine residues were placed at the ligation site (see below), also on the solvent-exposed surface. The only change made in the interhelical hydrophobic recognition surface was the replacement of asparagine with valine (N16V) to allow the possibility of autocatalysis through one- and/or two-stranded  $\alpha$ -helical template structure(s)<sup>17</sup>. The ligation site was chosen to lie on the solvent exposed surface of the  $\alpha$ -helical structure to avoid interference with the hydrophobic recognition surface. The amide-bond-forming process was based on the thioester-promoted peptide fragment condensation strategy of Kent<sup>19</sup> in order to circumvent potential side reactions which may arise from reactions at amino and carboxylic acid side-chain functionalities. The reaction was made up of an equal mixture of the electrophilic fragment (preactivated as the thiobenzyl ester COSBn) and the nucleophilic fragment, in the absence or presence of various amounts of template (Fig. 2). The coupling reaction was found to be remarkably regio- and chemo-selective, giving rise to less than 15% side products during the course of the reaction, most of which are due to the slow hydrolysis of the thioester fragment. The reaction products were identified by either direct isolation from reaction mixtures followed by characterization and sequencing by mass spectrometry, and/or by comparison with authentic samples using HPLC co-injections (Fig. 3).

Autocatalysis in template production is unequivocally established when reaction mixtures differing only in the initial concentration of the template are studied (Fig. 4a). Marked increases in the initial rates of product (template) formation are observed as the initial concentration of the template is increased. The increase in the initial rates of product formation correlates with the square root of the initial template concentration (Fig. 4b). The observed square-root rate profile, which has also been noted in previously characterized synthetic replicators, presumably reflects catalyst (template) inhibition through aggregation<sup>7-13</sup>. The experimental data sets (three sets of five parallel reactions) were also analysed according to empirical rate equations of von Kiedrowski using the program SimFit<sup>16</sup>. These analyses indicate an apparent autocatalytic rate constant  $k_a = 29.4 \pm 0.8 \text{ M}^{-3/2} \text{ s}^{-1}$  and the background rate constant  $k_b = 0.063 \pm 0.008 \text{ M}^{-1} \text{ s}^{-1}$  giving an autocatalytic efficiency ( $\epsilon = k_a/k_b$ ) of approximately 500 (ref. 20). We note that in efficient autocatalytic processes, product formation is expected to display a sigmoidal growth pattern, especially for reactions in which no template is initially present<sup>20</sup>. In this study, sigmoidal growth of template is also noticeable (Fig. 4a). As a control when similar reactions were performed in the

FIG. 1 Diagram of the minimal autocatalytic reaction cycle in the self-replicating  $\alpha$ -helical peptide. It consists of two reactive peptide fragments, the electrophile (blue) and the nucleophile (red), each identical in sequence to their putative complementary recognition sites on the template chain (grey). Pre-organization of the reactants on the template, which is mediated by specific inter-helical hydrophobic interactions, promotes peptide fragment condensation. The product, which is an identical copy of the template itself, then becomes part of the autocatalytic cycle to further promote peptide fragment condensation. The single-stranded peptides are depicted as partially unfolded or random coil structures.

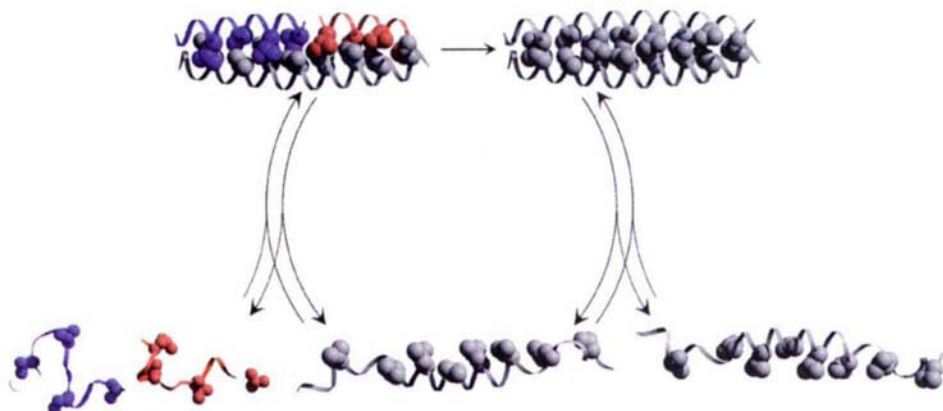


FIG. 2 Helical wheel diagram of the template peptide in the dimeric  $\alpha$ -helical coiled-coil configuration emphasizing the heptad-repeat motif (top) and the peptide sequences employed in this study (bottom). The interhelical recognition surface is dominated by hydrophobic packing interactions (positions a and d) and electrostatic interactions (positions e and g). Placement of a single charged polar residue at positions a or d destabilizes the coiled-coil structure by disrupting helix-packing interactions (design of the two crippled templates are based on this principle). Amino acids at positions b, c and f lie on the solvent-exposed surface of the helical structure and do not participate in the molecular recognition processes (arrows indicate the position of the cysteine residue on the nucleophilic peptide fragment and the thioester-activated carboxy terminus of the electrophilic peptide fragment). In the course of the reaction, the electrophilic fragment undergoes an attack by the amino-terminal sulphhydryl functionality of the nucleophilic fragment to give the corresponding cysteine thioester intermediate, which rapidly rearranges to give the thermodynamically favoured amide bond at the ligation site. Tyrosine residues were incorporated on the solvent-exposed surfaces to allow accurate determination of peptide concentrations. Furthermore, the template and the electrophilic peptide fragments were acylated at the N termini with 4-acetamidobenzoic acid (Ar) to allow sensitive monitoring of product formation by high-performance liquid chromatography (HPLC).

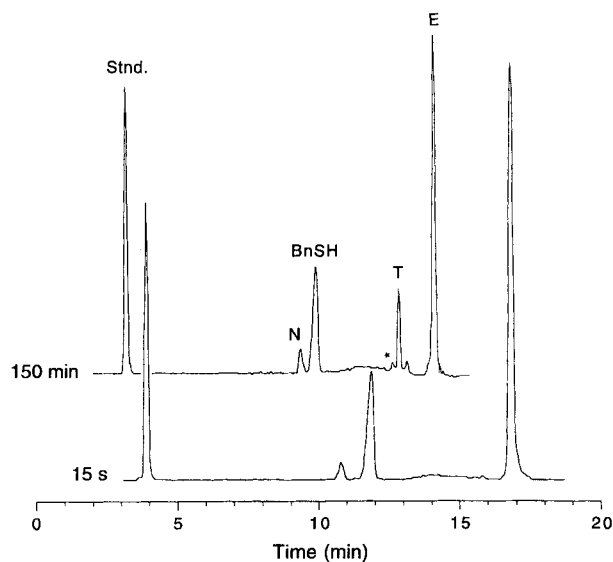
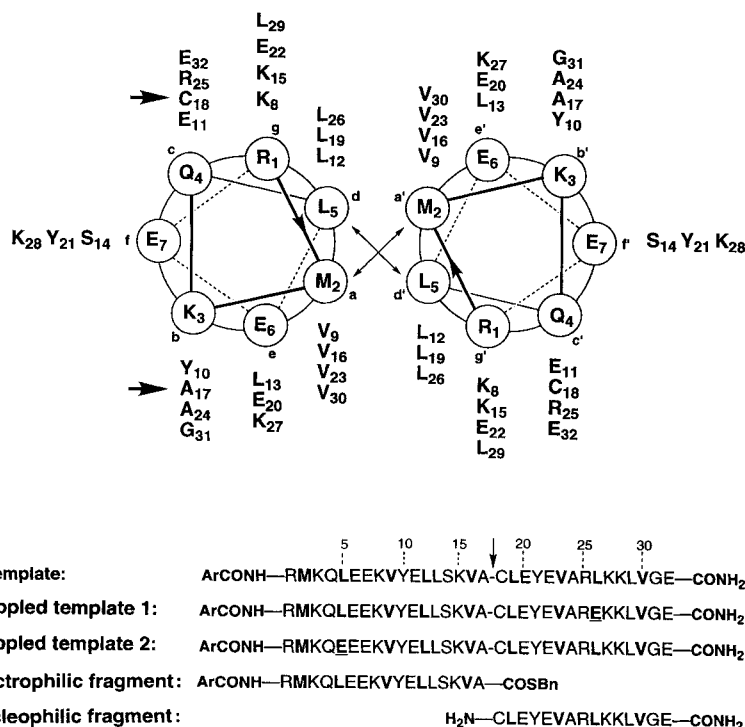


FIG. 3 Reverse-phase HPLC reaction profiles, monitored at a wavelength of 270 nm, 15 s (bottom trace) and 150 min (top trace) after the initiation of the reaction (\* denotes the hydrolysis product, N denotes the nucleophile, T the template, E the electrophile and BnSH is benzylmercaptan). **METHODS.** All reactions were started by adding benzylmercaptan-saturated MOPS buffer (final concentration: 154 mM, pH 7.50) to an acidic solution containing equivalent amounts of electrophilic and nucleophilic peptides (final concentration: 90  $\mu$ M) to give a total volume of 500  $\mu$ l. Temperature was maintained at 21 °C. Samples (50  $\mu$ l) were quenched with 2% aqueous trifluoroacetic acid (70  $\mu$ l) and analysed by reverse-phase HPLC at 270 nm. Concentrations were determined relative to 4-acetamidobenzoic acid as the internal standard (stnd).

presence of guanidinium hydrochloride, which prevents structural organization, no template catalysis was observed (Fig. 4c).

*A priori*, there are at least four pathways that could lead to product formation—three of them constitute the autocatalytic channel (Fig. 5). The ternary complex (Fig. 5a) where both reactants are recognized by the template is defined as the 'true' self-replication intermediate. The binary complexes (Fig. 5b, c) are also plausible autocatalytic intermediates but do not mediate self-replication as only one of the reactants is recognized by the template. To probe the contribution, if any, of these two binary complexes to the overall autocatalytic process, two 'crippled' templates<sup>21,22</sup> were designed, synthesized and characterized (Fig. 2). With respect to the normal template, each of the crippled templates carries a single glutamic acid residue mutation in its hydrophobic recognition surface which severely disrupts interhelical recognition and association. The crippled templates 1 and 2 (Fig. 2) were designed to express the disruptive mutation at the recognition surfaces where the putative binding sites of the nucleophilic and the electrophilic fragments reside, respectively. Therefore, when reactions are performed in the presence of added crippled templates 1 or 2, any contribution to the overall rates of product formation can only come from the intermediary of the binary complexes shown in Fig. 5b or c, respectively. When reaction mixtures differing only in the initial concentration of the crippled template were studied—identical reaction conditions to the ones described for the normal template—no increase in the initial rates of product (template) formation was observed (Fig. 4c). These control experiments strongly suggest that the contribution of the autocatalytic pathways (shown in Fig. 5b, c) to the overall product formation is insignificant, and the observed autocatalysis must in fact proceed through the self-replicative ternary intermediate (Fig. 5a) in agreement with the described rate analysis and reaction modelling. In addition, preliminary competition studies between the normal and Leu26Ala mutant (in which an alanine residue is substituted for a leucine at position 26) nucleophilic peptide fragments, performed in the presence and/or absence of normal as well as the corresponding mutant templates, have indicated that even such a conservative mutation



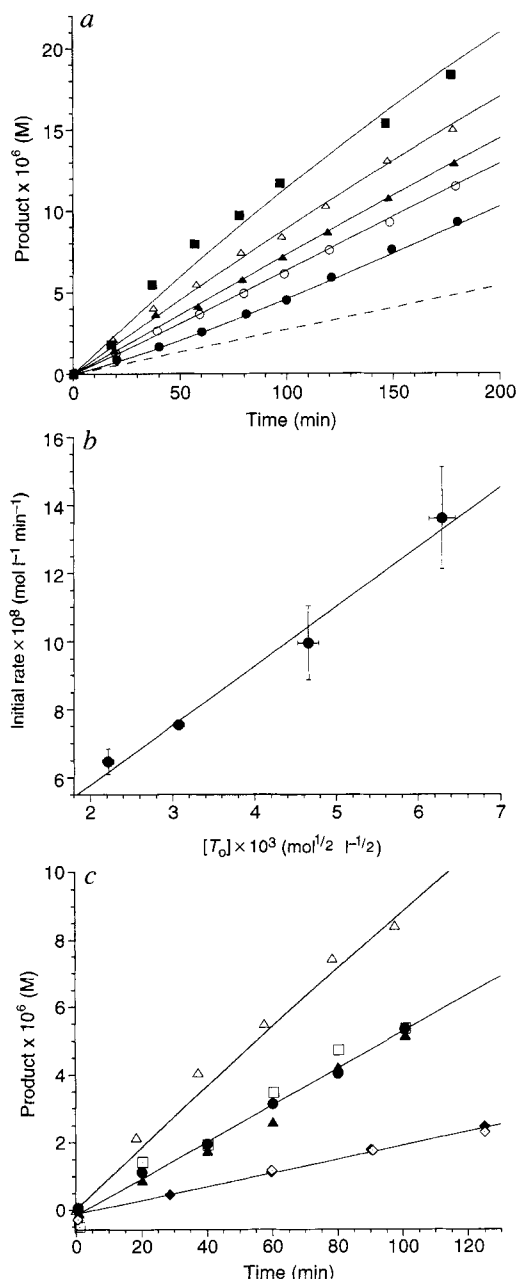


FIG. 4 a, Template production as a function of time for reaction mixtures containing different initial concentrations of template. (filled circles) In the absence of any added template, and in the presence of (open circles) 5 μM; (filled triangles) 10 μM; (Δ) 20 μM; and (filled squares) 40 μM initial template concentration. Curves were generated by nonlinear least-squares fit of the data to the empirical rate equation of von Kiedrowski using the program SimFit. The rate equation  $dT/dt = (N_0 - T)(E_0 - T) / [(k_a(T + T_0)^{0.5} + k_b)]$  describes the initial rate of template formation in two terms: a template-independent term (background) with the apparent rate constant  $k_b$  and a template-dependent term (autocatalytic) with a rate constant  $k_a$ .  $N_0$ ,  $E_0$ , and  $T_0$  are the initial concentrations of the nucleophile, electrophile and the template, respectively. All reactions were repeated three times (one set is shown) and simulated separately. The dashed line represents the calculated production of template in the absence of autocatalysis. b, Initial rate of template production versus the square root of the initial template concentration. Error bars show standard deviations of three independent runs. c, Template production as a function of time in the presence of 20 μM normal template (open triangles); 20 μM crippled template 1 (open squares), and 20 μM crippled template 2 (filled triangles). The template production is also shown for reactions carried out in pH 7.5 buffered solution of 4.3 M guanidinium hydrochloride in the absence (open diamonds) and the presence of 20 μM normal template (filled diamonds).

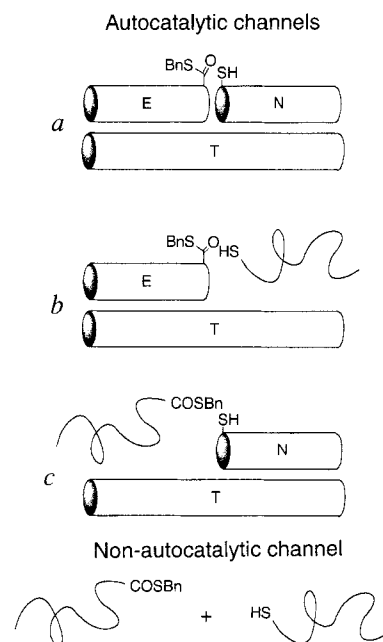


FIG. 5 Schematic representation of various plausible pathways in template production. The autocatalytic channel can be composed of three pathways: reactions can proceed through the intermediary of the ternary complex (a) and/or binary complexes (b and c). These two binary complexes have been shown not to be involved in the present study (see text). Mediation through intermediates of higher aggregation states is however plausible but seems to give a negligible contribution under the reaction conditions employed, based on the kinetic analyses and reaction modellings (see text). The non-autocatalytic channel (background) is probably dominated by a bimolecular event, although unimolecular pathways proceeding through pre-association of the electrophilic and nucleophilic peptide segments is also plausible. However, no noticeable aggregation of the nucleophilic and electrophilic fragments could be detected by circular dichroism spectroscopy even at approximately twofold higher concentrations than used here. Asterisks represent the approximate location of glutamic acid mutations on the helix-recognition surfaces of the crippled templates.

can abolish the self-replication process. Together, these experiments suggest implicitly that sequence-selective molecular recognition on complementary surfaces has a pivotal role in peptide self-replication.

The data presented here establish the chemical feasibility of peptide self-replication. Although the system described is based on  $\alpha$ -helical coiled-coil structures, peptide self-replication through the intermediary of larger helical aggregates as well as  $\beta$ -sheet-containing motifs are also likely<sup>23,24</sup>. Furthermore, on the basis of the large number of natural enzymes that are made up of multiple domains, and of recent discoveries indicating that domain swapping is a functional mechanism for interconversion between monomers and oligomeric forms (ref. 25 and refs therein), we suggest the possibility of protein self-replication in which the catalytic activity of the enzyme could also be conserved. Given that amino acids and polypeptide species could have been produced under prebiotic conditions, the possibility of self-replicating peptide sequences should be considered in the early evolution of living systems. □

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## Trace gas emissions on geological faults as indicators of underground nuclear testing

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UNDERGROUND nuclear explosions produce trace amounts of distinctive but ephemeral radionuclide gases. In the context of monitoring a comprehensive test ban treaty, the detection of these gases within the territory of a signatory, during a challenge inspection<sup>1–5</sup>, may indicate the occurrence of a clandestine nuclear event. Here we report the results of an experiment simulating a well-contained underground nuclear explosion, undertaken to test the ability of natural gas-transport processes to move highly dilute and rapidly decaying radionuclides to the surface. We find that trace gases are transported to the surface within periods of weeks to a year, by flow along faults and fractures driven by barometric pressure variations. Both our observations and related simulations exhibit a chromatographic behaviour, with gases of higher atomic mass and lower diffusivity reaching the surface more rapidly. For a 1-kilotonne nuclear test under conditions identical to those of our experiment, we predict that short-lived <sup>133</sup>Xe and <sup>37</sup>Ar would be detectable, respectively, about 50 and 80 days after the detonation. Our results indicate that radionuclide sampling along natural faults and fractures, as a forensic tool, can be an extremely sensitive way to detect nearby underground nuclear explosions that do not fracture the surface.

Sited at a depth of 400 m in the bedded tuffs<sup>6</sup> of Rainier Mesa (Fig. 1a) at the Nevada Test Site, a simulated 1-kilotonne (1-kt) nuclear explosion was produced by the detonation of 1.3 million kilograms of chemical explosives in a mined cavity on 22 Septem-

ber 1993. At this depth, the overburden is more than adequate to contain such an explosion. Whereas a somewhat shallower detonation might have produced a collapse crater or extensive fractures connecting the cavity with the surface, this explosion produced no visible structural changes including fissuring at the surface<sup>7</sup>. Such an event represents an extreme challenge for the application of on-site forensic techniques which are intended to provide evidence of the violation of a nuclear treaty. Of these techniques, the sampling of soil-gases for rare, explosion-produced radionuclides at the surface near a suspected underground test is particularly attractive because it is minimally invasive and yields relatively unambiguous results about the occurrence of an event. Our experiment was designed to evaluate the ability of transport processes to carry dilute and rapidly decaying radionuclides from the rubble-detonation site to the surface within the limited period of detectability of these gases.

A gas bottle containing 1.3 m<sup>3</sup> (STP) of <sup>3</sup>He was placed in the cavity before loading the explosives. Another bottle with 8 m<sup>3</sup> (STP) of sulphur hexafluoride (SF<sub>6</sub>) was positioned just behind a metal bulkhead at the entry to the cavity to minimize any thermal degradation of the tracer although the gas is somewhat resistant to thermal breakdown<sup>8</sup>. Following detonation and release of the tracers, gas chromatography was used to detect the heavy SF<sub>6</sub> gas (relative molecular mass 146) in air samples which have a low background level of less than 3 parts per trillion by volume (p.p.t.v.) at the test site. Similarly, the low atmospheric background level (7.34 p.p.t.v.) of <sup>3</sup>He (atomic mass 3) permits a very low threshold for detection using mass spectrometry.

Almost 200 gas samples were obtained over approximately 500 days during which a wide variety of barometric events were recorded by a weather station on Rainier Mesa (elevation 2,286 m above sea level). Most of the sampling occurred in the autumn and spring, when large barometric depressions occur.

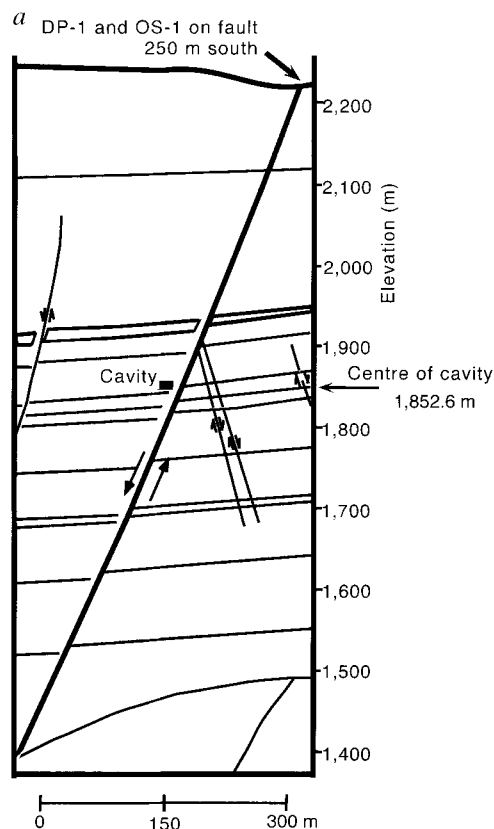


TABLE 1 Summary of Rainier Mesa gas sampling observations

| Sample suite | Date (d/m/y) | No., type of site | Total detects (total samples) | Locations of SF <sub>6</sub> detection | Detected SF <sub>6</sub> concentration (p.p.t.v.) | Locations of <sup>3</sup> He detection | Detected <sup>3</sup> He concentration (p.p.t.v.) | Barometric pressure (mbar) |
|--------------|--------------|-------------------|-------------------------------|----------------------------------------|---------------------------------------------------|----------------------------------------|---------------------------------------------------|----------------------------|
| 1            | 8/7/93       | 3,F               | 0 (3)                         |                                        | ND                                                |                                        | —                                                 | 772.1                      |
| 2            | 11/8/93      | 3,F               | 0 (5)                         |                                        | ND                                                |                                        | ND                                                | 775.2                      |
| 3            | 9/9/93       | 3,F               | 0 (6)                         |                                        | ND                                                |                                        | ND                                                | 777.6                      |
| 4            | 22/9/93      | Tunnel portal     | 0 (2)                         |                                        | ND                                                |                                        | ND                                                | 772.5                      |
| 5            | 24/9/93      | 3,F               | 0 (3)                         |                                        | ND                                                |                                        | —                                                 | 778.2                      |
| 6            | 10/11/93     | 3,F               | 1 (6)                         | OS-6                                   | 340                                               |                                        | ND                                                | 764.8                      |
| 7            | 17/3/94      | 3,F               | 3 (7)                         | OS-1, OS-6*                            | 540, 580, 280                                     |                                        | ND                                                | 771.2                      |
| 8            | 23/3/94      | 3,F               | 3 (6)                         | OS-1, OS-2, OS-3                       | 580, 450, 400                                     |                                        | ND                                                | 765.3                      |
| 9            | 17/5/94      | 2,F               | 0 (2)                         |                                        | ND                                                |                                        | —                                                 | 765.2                      |
| 10           | 19/5/94      | 3,F               | 0 (3)                         |                                        | —                                                 |                                        | ND                                                | 770.7                      |
| 11           | 11/8/94      | 1,F               | 0 (1)                         |                                        | ND                                                |                                        | —                                                 | 779.1                      |
| 12           | 29/9/94      | 5,F               | 0 (9)                         |                                        | ND                                                |                                        | ND                                                | 771.6                      |
| 13           | 4/10/94      | 5,F               | 0 (11)                        |                                        | ND                                                |                                        | ND                                                | 766.6                      |
| 14           | 6/10/94      | 6,F               | 2 (11)                        | OS-1                                   | 13                                                | OS-6                                   | 8.42                                              | 771.2                      |
| 15           | 12/10/94     | 6,F               | 1 (12)                        | OS-3                                   | 18                                                |                                        | ND                                                | 769.8                      |
| 16           | 2/11/94      | 6,F               | 3 (15)                        | DP-1, OS-6                             | 45, 45                                            | DP-1                                   | 9.22                                              | 760.9                      |
| 17           | 3/11/94      | 8,F               | 0 (15)                        |                                        | ND                                                |                                        | ND                                                | 764.5                      |
| 18           | 10/11/94     | 13,S; 6,F         | 3 (41)                        | DP-1, TP-4(S)                          | 18, 9                                             | DP-1                                   | 21.4                                              | 760.8                      |
| 19           | 16/11/94     | 18,S; 8,F         | 1 (40)                        |                                        | ND                                                | DP-1                                   | 14.7                                              | 762.8                      |

Multiple gas samples were obtained from sites on Rainier Mesa at the Nevada Test Site on at least 19 separate occasions starting before the underground detonation and continuing for over 400 days after it. Both diagnostic modelling<sup>5,10</sup> and some previous field work (R. H. Nilson *et al.*, unpublished results) strongly indicated that deeper, barometric lows, which are associated with storms, enhanced the probability of detecting gases at the surface. Thus, our data tends to be taken during such lows. (Owing to the poor accessibility of Rainier Mesa during storms, sampling by a two-man team tended to be limited by the onset of snowfall.) Sites on faults and fractures were also preferred in view of the previous work. Here a site is classified as being either on a fracture/fault (F in column 3) or on a soil site that had been covered by a tarpaulin to trap the gas (S). Only one S-type site, TP-4, not obviously associated with a fault or fracture, yielded a value of SF<sub>6</sub> above the background. In the fourth column the total number of detections is indicated along with the total number of samples taken on a given day. The locations where detections occurred are given in the fifth and seventh columns, along with the respective concentrations of SF<sub>6</sub> and <sup>3</sup>He (sixth and eighth columns) that were obtained from those sites; the symbol 'ND' indicates that excess concentrations were not detected, and a dash indicates that no analyses were performed. An average barometric pressure is given for each sampling suite in the last column. For comparison, the average barometric pressure for the entire pressure history is 773 mbar.

\* Two samples were obtained from OS-6, separated by several hours: the first yielded a concentration of 580 p.p.t.v. and the second, 280 p.p.t.v.

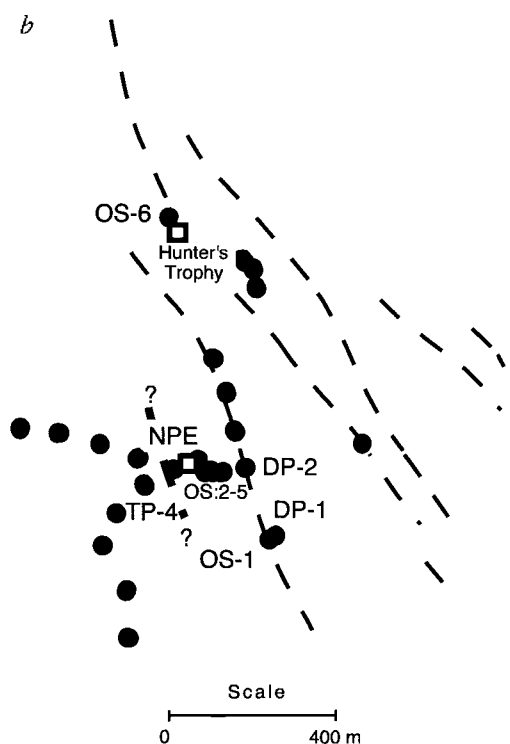
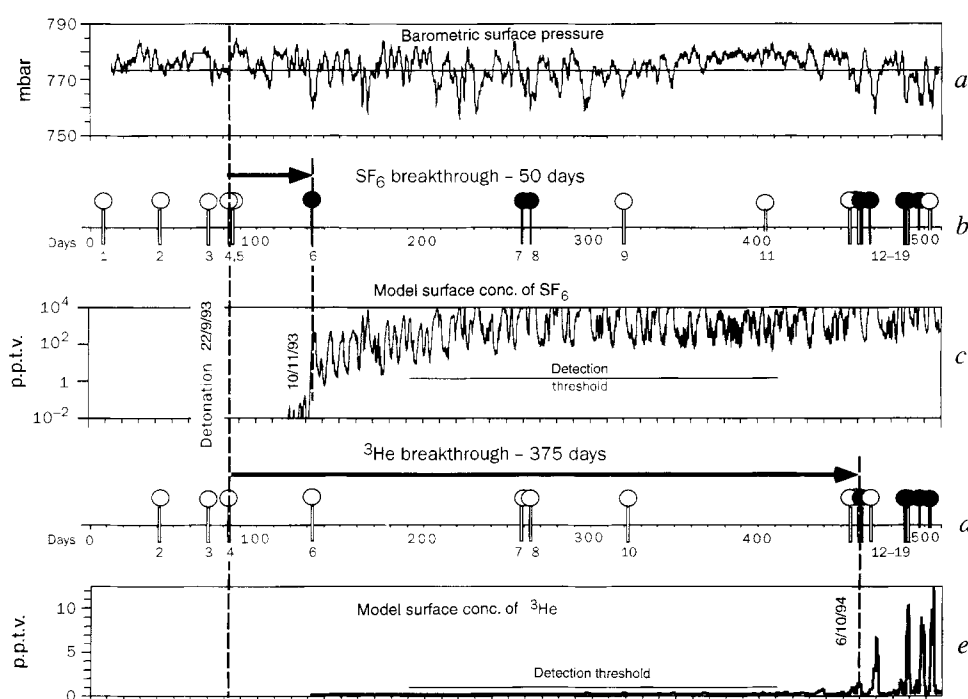


FIG. 1 a, Cross-section of layered tuffs of Rainier Mesa at the Nevada Test Site showing the location of the cavity relative to the surface and surrounding faults including the main fault on which most prolific sample stations DP-1 and OS-1 (Fig. 1b) were situated. These stations are several hundred metres from the point on the fault which is nearest to surface ground zero. (Figure adapted from ref. 6). b, Plan view of the distribution of sample points (filled circles) relative to existing faults (dashed lines) and surface ground zero of the detonation (empty square labelled NPE) which has become known as the Non-Proliferation Experiment (NPE). The most prolific site, DP-1, is located on the fault that is thought to pass close to the detonation cavity. Upon emplacement (2 November 1994), it immediately provided the highest concentrations of SF<sub>6</sub> obtained during the final sampling period (autumn 1994), and the highest <sup>3</sup>He concentrations overall. Large SF<sub>6</sub> values, in the 500–600 p.p.t.v. range, were obtained at OS-1 which is only a few metres from DP-1 and is in the fault zone. OS-1 was the first station to be emplaced. High values were also obtained along a fault ~550 m (1.3 cavity depths) north of the NPE surface ground zero. This site (OS-6), near an underground nuclear test called Hunter's Trophy, has produced the earliest detections of both tracers with the other sites, closer to the NPE, yielding detections shortly thereafter. (On the date <sup>3</sup>He was first detected, a site on the fault near OS-1 produced a concentration for <sup>3</sup>He that fell just below the 1 p.p.t.v. excess required to declare a detection.) A site marked 'TP-4' is one of ~15 stations surrounding the NPE surface ground zero where a plastic tarpaulin was laid out on the soil to trap gas as it reached the surface (all other unlabelled points are such 'tarpaulin' stations). Of all measurements from these tarpaulin sample points, only TP-4 once yielded an SF<sub>6</sub> concentration above background. All sites labelled 'OS' (Original Site) and 'DP' (Drive Point) are located on pre-existing faults or fissures. The former have sample tubes that were driven to 1–2 m depths manually, while the latter have sample tubes or drive points installed to depths of 5 m using a truck-mounted hydraulic ram.

FIG. 2 Sampling histories for both tracers ( $\text{SF}_6$  and  $^3\text{He}$ ), along with models of near-surface-concentration histories, plotted together with the history of the driving barometric pressure variations (a). The horizontal line indicates the average pressure, 773 mbar, over the sampling period. In b and d the suite of samples taken during a given barometric low is summarized either as a 'detect' (at least one sample of a suite above background; shown as a filled circle on a vertical bar) or a 'no detect' event (empty circle on a vertical bar) with the numbers beneath the symbols corresponding to the sample suite numbers of Table 1. Numerical models were used to produce plots (c,  $\text{SF}_6$ ; e,  $^3\text{He}$ ) of the tracer concentrations in excess of background values in p.p.t.v. at STP as functions of time. For reference, the thresholds of detection are given by the horizontal lines in plots. The concentration of  $\text{SF}_6$  is plotted on a log-linear scale because of the wide variation in values. The model exhibits a large change in concentration at the surface at a time corresponding to observations of the apparent arrival of  $\text{SF}_6$  (b). For  $^3\text{He}$ , the apparent arrival as interpreted from the observations (d) is also in excellent agreement with the linear plot of concentration as a function of time (e) as indicated by the model. (Note: two null events ('no detect') immediately precede the arrival ('detect') although on the scale of the diagram they appear as one null event.) Hydrological parameters used in the models, such as gas-phase porosity (0.10), permeability ( $10^{-15} \text{ m}^2$ ), fracture separation (6.4 m) and tortuosity (0.1), are thought to be appropriate in a bulk sense for the fractured tuffs of Rainier Mesa and nearby Yucca Mountain with its well studied hydrological system. An integrated finite-difference, multiphase, flow and transport computer program<sup>13</sup> performs the gas transport simulations on a 4,200-element, 6.4 m wide by 400 m deep biased grid using the hydrological parameters as input. The



same fracture-matrix model is used for both the  $\text{SF}_6$  and  $^3\text{He}$  concentration histories; only the diffusivities and initial concentrations are different. The initial concentration distribution of  $\text{SF}_6$  and  $^3\text{He}$  occupies a region or 'halo' in the fractured porous medium extending upwards to 200 m beneath the surface of the mesa. This fracture model is consistent with models used in detonation containment studies<sup>10</sup>. Parameter sensitivity studies show that large changes in model parameters such as fracture width (0.001–0.004 m, 0.00125 m used), fracture separation (3.3–12.8 m, 6.4 m used), and initial 'halo' concentration of  $\text{SF}_6$  ( $3.0 \times 10^{-7}$  to  $2.2 \times 10^{-5}$ ,  $2.2 \times 10^{-5}$  mol tracer per mol air used) all result in changes to the arrival time of 13 days or less for  $\text{SF}_6$ .

Snow blocked access to the mesa during late autumn, winter and early spring. Typically, three or more stations were sampled during a barometric event. At some stations, stainless-steel tubes with porous end caps were driven into the ground to depths of 1.5–5 m along fractures and faults (Fig. 1b). The ground around these tubes was often covered with plastic sheeting to limit atmospheric infiltration. At other stations (TP-4 and unlabelled sites in Fig. 1b), located more or less radially from the point on the surface directly above the cavity or surface ground zero, sampling tubes were simply placed beneath plastic sheeting or tarpaulins which were spread on the ground to trap rising soil gases.

During a large barometric depression in the autumn of 1993 (Fig. 2a),  $\text{SF}_6$  was first detected at concentrations of 340 p.p.t.v. in fractures along a fault ~0.5 km or 1.3 overburden thicknesses from surface ground zero (sample site OS-6 in Fig. 1b and Table 1) 50 days after the detonation (Fig. 2b). After its arrival,  $\text{SF}_6$  was found to be well above background a number of times in later sample suites as indicated by Table 1 and the 'detect' symbols on the timeline (Fig. 2b). However, it is also evident that  $\text{SF}_6$  was not detected in other sample suites. The evanescent behaviour of the tracer as indicated by the 'no detects' after its initial detection may be the result of occasional near-surface dispersion due to winds, atmospheric infiltration and rain that results in additional sampling variability<sup>9</sup>. In addition, the diffusion of the tracer at depth as a result of its initial non-uniform distribution would be expected to give rise to a progressively weakening tracer signal, as discussed below.

Helium-3 was initially detected at levels exceeding two standard

deviations (taken conservatively to be 1 p.p.t.v. in excess of the 7.34 p.p.t.v. atmospheric level for  $^3\text{He}$ ) 325 days following the first detection of  $\text{SF}_6$  and 375 days after the detonation (Table 1 and Fig. 2d). It is interesting but not surprising that the first detected arrivals of  $^3\text{He}$  and  $\text{SF}_6$  occurred at the same sampling location, OS-6, which is not on the nearest fault to surface ground zero (Fig. 1b). It seems quite reasonable that gas-propagated fractures from the detonation could pump tracers into that fault, which dips downwards towards the detonation cavity. Several weeks later, another sample was obtained from a location (DP-1) on a fault passing much nearer the cavity (Table 1 and Fig. 1b) that yielded the maximum observed concentration of 21.4 p.p.t.v. A week following this maximum, the same location produced yet another measurement of 14.7 p.p.t.v., the second largest concentration of  $^3\text{He}$  obtained.

These observations cannot be explained by gaseous diffusion alone; a numerical model indicates that periods of tens to hundreds of years are required to produce detectable concentrations of  $\text{SF}_6$  at the surface. Furthermore, in a diffusively dominated transport environment, the much more diffusive  $^3\text{He}$  should arrive before  $\text{SF}_6$  which is not the case here. Finally, virtually all the samples yielding non-zero concentrations of the tracers are associated with pre-existing faults or surface fissures (DP-1, OS-1, OS-6) indicating an important role for these pathways in the transport process (Fig. 1b and Table 1).

Analytical and numerical models<sup>10</sup> of gas transport in a fractured porous medium subject to barometric surface-pressure variations indicate that the speed of transport along fractures over a vertical scale of hundreds of metres is orders of magnitude



greater than the diffusion rate. We used such a transport model that assumed vertical, millimetre-width fractures traversing the uniform porous matrix of the overburden into a region of uniform tracer concentration around the detonation point. In the model, flow along the fractures is driven by barometric pressure variations which were obtained from the Rainier Mesa weather station. Overburden properties were based on estimates thought to be appropriate for Rainier Mesa (see Fig. 2 legend). The initial concentrations of each tracer in the source region around the detonation point were determined by the volumes released in the explosion. The binary diffusivity, another parameter specific to each tracer, was also required in solving the advection–diffusion equations. The only free parameter was fracture width, which was adjusted to a value (0.00125 m) necessary to yield an  $^3\text{He}$  concentration similar to what was observed in the field at its apparent arrival time. With all the parameters thus fixed, the model was then used to calculate the concentration of  $\text{SF}_6$  at arrival, as well as its arrival time assuming the initial concentration and binary diffusivity appropriate for this tracer. Calculated arrival times for both tracers (Fig. 2c and e) seem to be in excellent agreement with the apparent (observed) arrivals. The calculated concentration of  $\text{SF}_6$  at arrival (Fig. 2c) is well within an order of magnitude of the observed values (Table 1).

Both the apparent and calculated arrivals are associated with deeper 'lows' of atmospheric pressure (a reduction of 1–2 kPa or 10–20 mbar). This is consistent with a recent containment-related study (R. H. Nilson *et al.*, unpublished results) that strongly correlates trace-gas arrivals with storm-related lows. An initially counterintuitive explanation for the difference between the apparent arrivals of the two tracers involves the eight-fold larger binary gas diffusivity of  $^3\text{He}$  compared to  $\text{SF}_6$  (ref. 11). In this fracture-matrix regime, the most diffusive gas arrives last at the surface according to our models. The high diffusion coefficient of helium actually impedes its transport along a fracture by allowing it to diffuse out of the vertical flow into the porous fracture walls far more effectively than  $\text{SF}_6$ .

The model assumption of a simple, initially uniform explosion-emplaced zone of gas about the detonation point, which is permeated by fractures, explains the growing concentration of the tracer with time following its calculated arrival (Fig. 2c). That the field observations seem to show a decay of concentration with time rather than growth (Table 1) is a result that can be modelled by a non-uniform source region consisting of thin, tracer-impregnated zones in the fracture walls that extend a portion of the fracture's length. Future modelling will address the issue of more realistic initial distributions of tracer or radionuclide concentration.

Given the very good agreement between our gas-transport model and the observations, we use it to predict the arrival of detectable concentrations of  $^{37}\text{Ar}$  and  $^{133}\text{Xe}$  assuming a 1-kt fission detonation. Argon-37 and  $^{133}\text{Xe}$  are preferred for the detection of nuclear explosions for two reasons: (1) they are not produced

naturally in significant quantities so that natural background levels are exceedingly low, and (2) their short half-lives of 34.8 days and 5.2 days, respectively, can be used to infer the recentness of an event. The model specific to argon included the concentration of  $^{37}\text{Ar}$  produced by fission-neutron bombardment of  $^{40}\text{Ca}$  in the soil; the diffusivity of argon, which is between that of  $^3\text{He}$  and  $\text{SF}_6$ ; its solubility in ground water and its half-life. The model predicts that 80 days after detonation the concentration of  $^{37}\text{Ar}$  at the surface would have produced decay rates in gas samples of  $6\text{Bq m}^{-3}$  (one becquerel (Bq) is one decay per second), which is readily detectable with current laboratory methods<sup>12</sup>. However, the decay of  $^{37}\text{Ar}$  to  $^{37}\text{Cl}$  will limit the sampling 'window' during which surface detection is possible. Using an analogous model for  $^{133}\text{Xe}$ , we found that its rather short half-life does not prevent it from reaching the surface in detectable quantities ( $41\text{Bq m}^{-3}$ ) about 50 days after detonation. The much larger initial quantity of  $^{133}\text{Xe}$  produced relative to that of  $^{37}\text{Ar}$ , combined with its lower diffusivity (which is comparable to that of  $\text{SF}_6$  causing it to be transported more rapidly than  $^{37}\text{Ar}$  to the surface), offsets its loss by rapid decay.

These predictions are likely to be conservative for two reasons. In our radionuclide transport simulations, we have assumed production rates that are low for both radionuclides:  $7 \times 10^{-5}\text{ mol}$  ( $9.7 \times 10^{12}\text{ Bq}$ ) of  $^{37}\text{Ar}$  per kilotonne, which is less than half of that produced by an explosion characterized by the intentionally high attenuation of fission neutrons<sup>13</sup> needed for  $^{37}\text{Ar}$  production, and  $7.7 \times 10^{-3}\text{ mol}$  ( $6.7 \times 10^{15}\text{ Bq}$ ) of  $^{133}\text{Xe}$  per kilotonne, corresponding to 50% of the fission chain yield of this isotope. Further, our predictive models for radionuclide transport are calibrated using the observed arrivals of the  $\text{SF}_6$  and  $^3\text{He}$  tracers rather than their actual arrivals. The observed arrivals of the two tracers can occur later than their actual arrivals owing to imperfect or sparse sampling. As a result, our models will tend to underestimate transport rates of trace gases through the overburden.

We conclude that even deep and very well contained underground nuclear explosions, which do not propagate fractures to the surface, are liable to detection by the barometrically driven emission of short-lived radionuclides from nearby surface-breaking faults, natural and mining-induced fracture networks and cooling joints which tend to be found<sup>14</sup> in less friable subsurface environments common in mining districts. Deep barometric lows associated with large storm fronts are needed to rapidly transport the higher-atomic-mass gases, which include the radionuclides of interest, to the surface. Thus, selecting the timing of a challenge inspection to include the arrival of weather fronts may be necessary to optimize the possibility of detection. Owing to the heterogeneity of pathways for gas transport, we found that it was a better strategy to locate sampling stations on existing faults and fractures, even at distances of hundreds of metres from surface ground zero, than to select a sampling station using proximity considerations alone. □

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# The first Cretaceous bird from Madagascar

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**We report the discovery of two exquisitely preserved specimens of a new, very primitive bird from the Late Cretaceous period of Madagascar. The new taxon, *Vorona berivotrensis*, is provisionally placed phylogenetically in an unresolved trichotomy with Enantiornithes and a clade consisting of *Patagopteryx* and Ornithurae. These specimens are the first known pre-Holocene birds from Madagascar and the first avian skeletal remains from the Mesozoic era of a large portion of Gondwana.**

Knowledge of the early evolution of birds is overwhelmingly dominated by fossils from northern continents. Important discoveries of Mesozoic birds from Gondwanan land masses over the past 15 years have begun to alter concepts regarding this history through the addition of several new higher taxa<sup>1,2</sup>. Nonetheless, for Gondwana there are no records of Jurassic birds, and only three occurrences of skeletal remains of Cretaceous birds outside South America<sup>3-5</sup>. Several footprints from Morocco constitute the only Mesozoic record of Aves from an enormous area encompassing Africa, Madagascar, the Indian subcontinent, and the mainland of Antarctica<sup>6</sup> (Fig. 1).

The new bird specimens described here were found during a joint SUNY Stony Brook–Université d'Antananarivo expedition to the continental Maevarano Formation (Upper Cretaceous), northwestern Madagascar, in 1995. The diverse fauna from this formation also includes fishes, frogs, turtles, snakes, lizards, crocodiles, sauropod and non-avian theropod dinosaurs, and mammals<sup>7-9</sup>.

Aves  
Metornithes<sup>10</sup>  
Ornithotharaces<sup>2</sup>

*Vorona berivotrensis* gen. et sp. nov.

**Etymology.** *Vorona* (voo-roo-na; Malagasy), bird; berivotra, referring to the village of Berivotra.

**Holotype.** Université d'Antananarivo (UA) 8651. Left distal tibiotarsus and complete tarsometatarsus found in articulation (Fig. 2).

**Referred specimen.** Field Museum of Natural History (FMNH) PA 715, associated complete right tibiotarsus, fibula, and femoral head (Fig. 3). FMNH PA 715 may be from the same individual as holotype but this is not demonstrable.

**Locality and horizon.** Holotype and referred specimen from Locality MAD93-18, Upper Cretaceous (Campanian) Maevarano Formation, Mahajanga Basin, near the village of Berivotra, Madagascar.

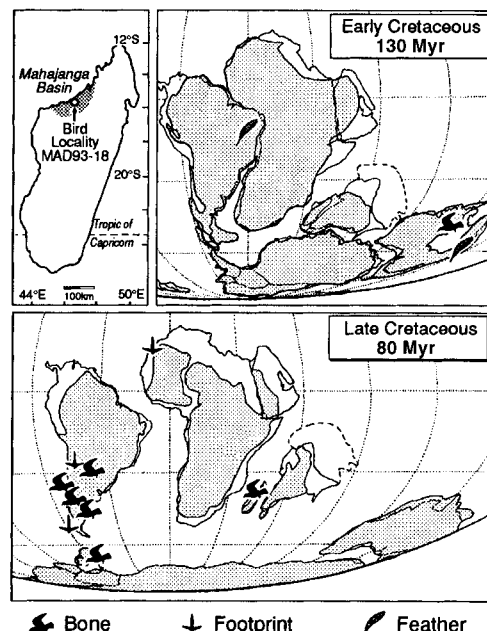
**Diagnosis.** Possesses short, blunt cnemial crest on tibiotarsus; irregular low ridge on medial surface of proximal tibiotarsus; narrow but deep notch on proximodorsal tarsometatarsus between metatarsals II and III; expanded vascular groove proximal to distal foramen on tarsometatarsus (Figs 2, 3).

The femur has a robust neck and round head (Fig. 3). The dorsomedial surface of the head is flattened and bears a broad, shallow depression for the capital ligament, a condition present in Enantiornithes and Ornithurae (Hesperornithiformes + Ichthyornithiformes + Neornithes) but unknown for non-avian

maniraptoran theropods<sup>1</sup>.

The proximal end of the long and slender tibiotarsus has a single very short, blunt, and robust cnemial crest (Fig. 3). Enantiornithines have either no distinct crest or a very small craniomedial crest. A prominent cranially projected cnemial crest is present in non-avian maniraptorans, whereas two distinct crests are typical of Ornithurae<sup>2</sup>. A well-developed, bulbous, lateral condyle for fibular articulation is present and a pronounced fibular crest extends down the proximal quarter of the shaft. On the medial surface of the proximal one-fifth of the tibiotarsus is an irregular low ridge not found in any other known Mesozoic bird.

The calcaneum and astragalus are fused to each other but only partially coossified to the tibia, as evidenced by a well-defined line of contact on the caudal, lateral, and medial aspects (Figs 2, 3). The proximal tarsals are unfused in non-avian maniraptorans, but are fused in all birds except *Archaeopteryx* and some alvarezsaurids. The tibiotarsus is completely fused in Enantiornithes, *Patagopteryx*<sup>11</sup>, and Ornithurae, but is unfused or partially fused in non-avian maniraptorans, *Archaeopteryx*, alvarezsaurids, and *Iberomesornis*<sup>1,2,12</sup>. The astragalus has a very tall, triangular, laterally placed ascending process, and is one-fifth the length of the tibia. The medial condyle is twice the width of the lateral condyle, a condition seen in *Patagopteryx* and Enantiornithes, but contrasting to the subequal condyles of Ornithurae. The condyles are separated by a narrow, deep intercondylar sulcus that opens



**FIG. 1** Maps showing fossil bird locality MAD93-18 in the Mahajanga Basin (shaded crescentic area) of northwestern Madagascar (top left), and distribution of Gondwanan landmasses and fossil remains of birds in the Early Cretaceous (top right) and Late Cretaceous (bottom; modified from ref. 1). No birds are known from pre-Cretaceous Gondwanan strata. Shaded areas on the Cretaceous maps indicate distribution of subaerially exposed land relative to current land mass outlines (modified from ref. 18). Dashed line indicates hypothetical extent of the Indian subcontinent before collision with Eurasia (modified from ref. 19). Madagascar, with the Indian subcontinent attached to its eastern coast, separated from Africa in the Late Jurassic period (160–150 Myr). Indo–Madagascar attained its current position relative to Africa in the Early Cretaceous (130–125 Myr), and rifted from Antarctica–Australia at about the same time. Separation of Madagascar from the Indian subcontinent did not occur until the Late Cretaceous, roughly 90–85 Myr<sup>20</sup>. The isolation of Madagascar thus predates the deposition of the Maevarano Formation by roughly 5–10 million years.

proximally into a deep fossa overhung by the median edges of both condyles. This condition is also present in Enantiornithes. The lateral condyle lacks any trace of a fibular facet.

The gracile fibula rapidly narrows to a very slender splint. Although incomplete distally, it clearly did not reach the distal end of the tibiotarsus as in *Archaeopteryx* and non-avian maniraptorans. Proximally, there is a small, laterally-directed tubercle for the *muscle iliofibularis* (Fig. 3). This lateral orientation is shared with *Mononykus*<sup>13</sup> and *Patagopteryx*, but contrasts with the caudal or caudolateral orientation of Ornithurae<sup>2</sup>. The proximal end is transversely compressed and, though medially concave, lacks the prominent medial fossa typical of non-avian maniraptorans.

The tarsometatarsus is slightly more than one-third the length of the tibiotarsus. Metatarsals (MT) II through IV are firmly coossified except along the mid-shaft of MT III and IV (Fig. 2). Metatarsal V is a very narrow, splint-like element that lies on the lateroplantar margin of MT IV. Metatarsals II–IV are co-planar throughout their length. The proximodorsal margin has a deep, narrow notch between MT II and III. Metatarsal II is dorsoplantarly expanded proximally, and its proximal plantar surface bears a blunt ridge reminiscent of the sharp edge seen in *Soroavisaurus*<sup>14</sup>. Metatarsal II is slightly shorter than metatarsal IV, which, in turn, is shorter than MT III. Metatarsal II is plantarly bowed and lacks

the dorsolateral tubercle present in Enantiornithes<sup>14</sup>. Metatarsal III is more slender than MT II and IV proximally, but expands distally to exceed them in width. A small proximolateral foramen is present between MT III and IV. A broad vascular groove on the dorsal aspect extends proximal to the distal vascular foramen, which opens distally rather than on the plantar aspect.

The trochleae of MT II–IV are equally spaced. The trochleae of MT II and III are square in distal view with well-formed ginglymi, whereas that of MT IV is rectangular in outline and not ginglymoid. The trochlea of MT II is significantly smaller than that of MT III, contrasting with the condition seen in Enantiornithes. Metatarsal III bears the largest trochlea and lacks the plantar projection of the medial rim seen in avosaurid Enantiornithes<sup>14</sup>.

*Vorona* exhibits some primitive characters common to non-avian maniraptorans and basal birds such as *Archaeopteryx* (for example, a single cnemial crest, persistent metatarsal V). However, it also presents derived characters in the femoral head, fibula, proximal tarsals, and tarsometatarsus that indisputably support its avian status (Fig. 4). The position of *Vorona* within Aves (Avialae of others<sup>10,13,15</sup>) is not well resolved owing, in part, to its fragmentary nature. It lies, in our analysis, in a trichotomy with Enantiornithes and the clade formed by *Patagopteryx* and Ornithurae (Fig. 4).

The discovery of *Vorona* provides an important geographical range extension for Mesozoic birds, and a significant addition to

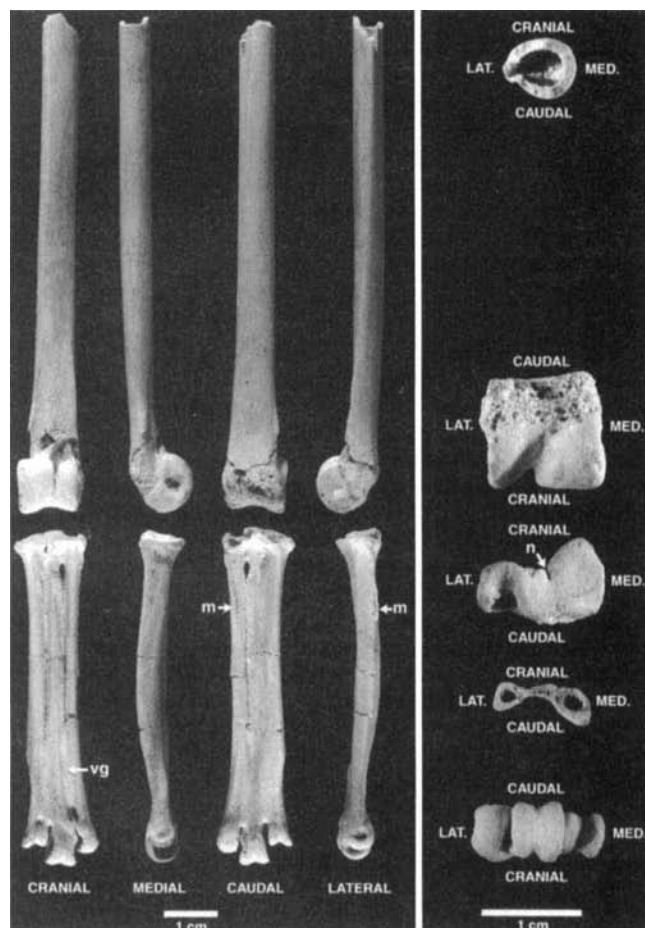


FIG. 2 Holotype specimen UA 8651; on the left are the left distal tibiotarsus (top) and complete tarsometatarsus (bottom) in (left and right) cranial, medial, caudal, and lateral views. On the right are (top to bottom): a cross-section of the tibiotarsus, the distal surface of the tibiotarsus, the proximal articular surface of the tarsometatarsus, a cross-section of the tarsometatarsus near mid-shaft, and a distal view of trochleae of the tarsometatarsus. Abbreviations: m, MT V; n, narrow but deep notch between MT II and MT III; vg, expanded vascular groove proximal to distal foramen.

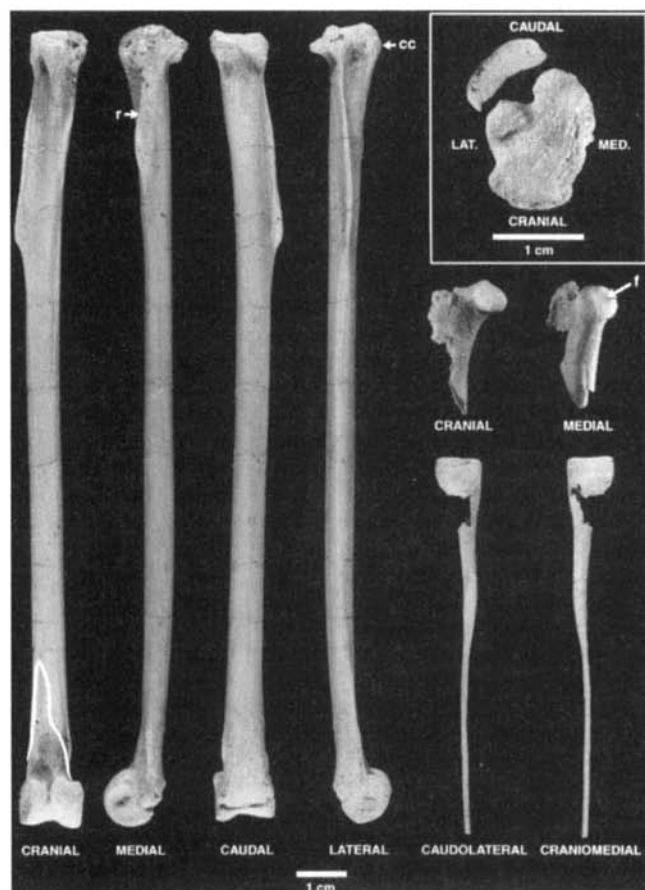


FIG. 3 Referred specimen FMNH PA 715; on the left is the right tibiotarsus in (left to right) cranial, medial, caudal and lateral views. The white line on the craniodistal tibiotarsus defines the extent of the ascending process of the astragalus. On the right are: the proximal articular surface of the fibula in articulation with the tibiotarsus (top, in box); the proximal right femur in cranial and medial view (middle); the right fibula in caudolateral and craniomedial views (bottom). Abbreviations: cc, short and blunt cnemial crest; f, fossa for the capital ligament; r, irregular low ridge.

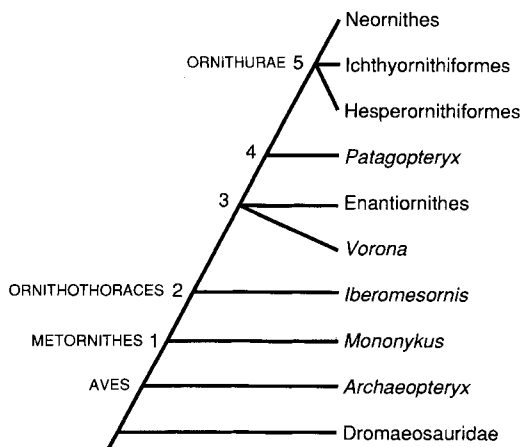


FIG. 4 Cladogram showing placement of *Vorona* within Aves. The preserved character states of *Vorona* were scored into the data matrix (Appendix 2) of ref. 21 with the following modifications: (1) characters 36, 59, 63 and 69 were transformed into multistate characters, (2) *Concornis* was included within Enantiornithes<sup>21</sup>, (3) *Mononykus* was added to the ingroup taxa, (4) characters 72, 73, 74, 75, 77, 78, 80, 81 and 84 were eliminated as they occur only in Enantiornithes, (5) on the basis of new evidence from recently prepared specimens, character 79 was rescored for *Patagopteryx* from 0 to 1. Two characters were appended to this data set: character 3 (Appendix 3 of ref. 21) and one new character (narrow, deep intercondylar sulcus on tibiotarsus). The resulting 77 character data set was processed using the implicit enumeration option (Hennig 86; steps = 106, consistency index = 0.77, retention index = 0.83). Given this limited data set (both in taxa and characters), the unambiguous synapomorphies diagnosing the nodes are: NODE 1: prominent ventral processes on cervico-dorsal vertebrae, ossified sternal keel, carpometacarpus, fused pelvic elements, prominent antitrochanter, pubic foot absent, trochanteric crest, caudal trochanter absent, fibula greatly reduced; NODE 2: pygostyle, strut-like coracoid, scapula with sharp caudal end, humerus shorter than ulna, shaft of radius thinner than ulna; NODE 3: synsacrum formed by more than 8 vertebrae, heterocoelus cervical vertebrae, scapulocoracoid articulation well below shoulder end of coracoid, humerus with well developed transverse ligamental groove, distal tarsals completely fused to metatarsals; NODE 4: sagittally curved scapula, extensor canal on tibiotarsus; NODE 5: quadrate orbital process pointed, articular pneumatized, fewer than 11 dorsal vertebrae, ossified uncinat processes, procoracoid process, humeral head globous, small acetabulum, pubis parallel to ilium, pubic shaft laterally compressed, prominent patellar groove, cranial cnemial crest, tubercle for *m. iliofibularis* caudolaterally or caudally directed, proximal metatarsal III plantarly displaced, well-developed tarsometatarsal intercondylar eminence.

the diversity of early birds<sup>16</sup>. Previously, the earliest established record of Malagasy birds was from the early Holocene epoch<sup>17</sup>. *Vorona* bears no close phylogenetic relationship to the extant and recently extinct malagasy avifauna, all of which belong to modern higher groups (Neornithes). □

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## Nautilus and the art of metabolic maintenance

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THE cephalopod mollusc *Nautilus* is often called a living fossil because its multichambered shell resembles that of extinct forms from the early Palaeozoic era. Living at depths of 100–300 m, the animals not only encounter zones of low oxygen concentration, but also exploit them for refuge or feeding<sup>1,2</sup>. Despite some modest recruitment of anaerobic sources of energy, *Nautilus* is able to survive severe bouts of hypoxia, mainly through its prodigious capacity for aerobic metabolic rate suppression. Here we show that the hypometabolic, hypoxic animal conserves energy further by means of prolonged ventilatory and circulatory pauses, during which time the blood, having a comparatively high oxygen affinity, is apparently loaded at the venous side, across the superficially located and voluminous vena cava. Under these highly arrested conditions, a significant fraction of the animal's aerobic metabolic rate can be accounted for by a slow 'metering out' of the O<sub>2</sub> store contained in the shell, indicating that the buoyancy chambers of *Nautilus* may occasionally subserve the role of a 'SCUBA tank'. The internal shell morphology and siphuncular arrangement seen in the fossilized remains of ammonites suggest that similar processes may have occurred.

*Nautilus pompilius* L. living at depths of 225–300 m were taken in baited traps from the sunken barrier reef south-east of Port Moresby, Papua New Guinea, and transported to the Motupore Island research station where they were acclimated to experimental temperatures (18 °C) which approximate those of their 'vertic niche'<sup>1</sup>. Unlike other cephalopod molluscs, *Nautilus* is very tolerant to hypoxia<sup>2</sup>. In the short term, this might be expected of any mollusc that bases its defensive strategy on withdrawal into a shell (to out-wait a persistent predator). But *Nautilus* can also remain active for long periods of time as it travels through waters of decreasing oxygen content. It does so by progressively depressing its metabolic rate to match the amount of ambient O<sub>2</sub> available (Fig. 1). *Nautilus* can also survive for at least a day in the most severe hypoxic environments that are ever likely to be encountered naturally<sup>2</sup>, by suppressing its aerobic metabolic rate to 4–8% of the level seen at normal oxygen concentrations (Fig. 1). These rates are equivalent to the hypometabolic feats of well-known 'facultative anaerobes'<sup>3,4</sup>. This represents a true metabolic suppression (that is, a decrease in heat production), because the estimates of ATP turnover in severe hypoxia, calculated from the aerobic metabolic rate and the accumulation in muscle of

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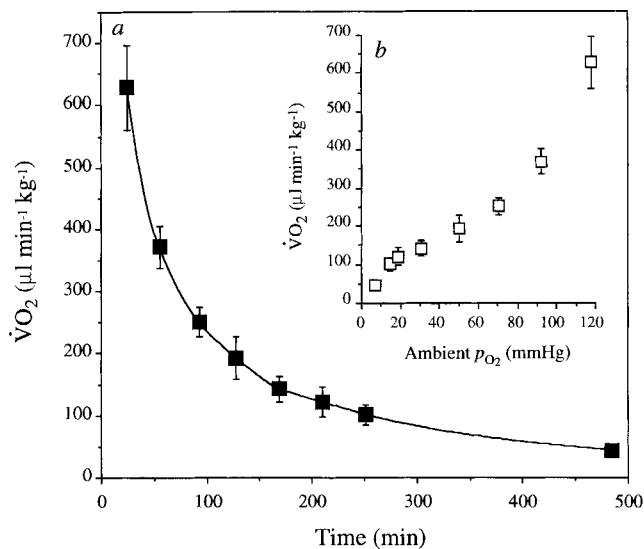


FIG. 1 a, Oxygen consumption ( $\dot{V}O_2$ ) during progressive hypoxia. b, Oxygenation response to lowered ambient partial pressures of oxygen ( $p_{O_2}$ ). Oxygen uptake was measured at 18 °C in a thermostatted, gas-tight, 5-litre perspex respirometer, using an EIL 7130 oxygen electrode and Kent  $O_2$  meter. Circulation within the tank was maintained by a magnetic stirrer.  $p_{CO_2}$  of the closed water system rose by 2–3 mmHg during the course of the experiments. Values are means (error bars s.e.m.) of experiments on 6 animals whose body weights were between 450 and 650 g, corresponding to flesh weights of 300–450 g.

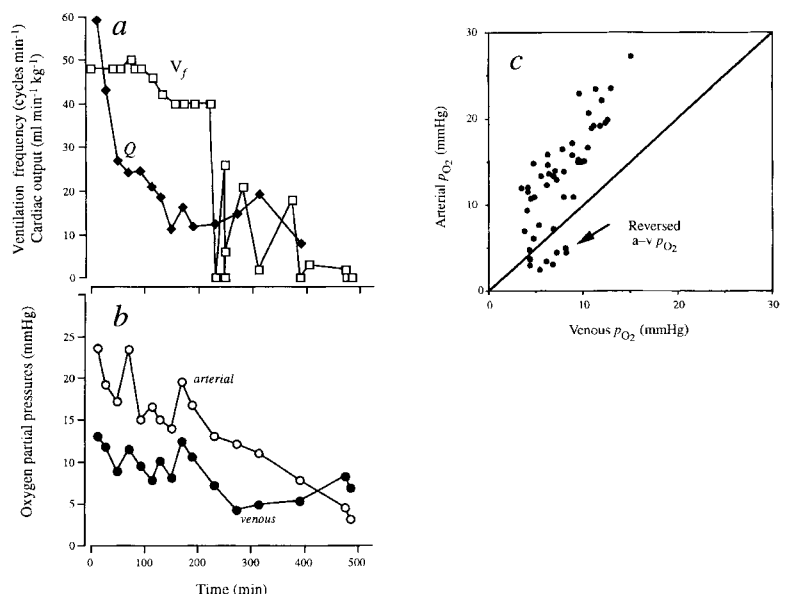
anaerobic metabolic end-products (Table 1), indicate that the recruitment of anaerobiosis makes up for less than 5% of the energy deficit arising from the lack of oxygen.

With such vast reductions in metabolic rate comes a decrease in activity, with brief periods of jet propulsion punctuating longer periods at rest<sup>2</sup>. During these inactive periods, ventilatory movements may cease altogether, cardiac output is dramatically reduced (Fig. 2), and the heartbeat can slow to one or two cycles per min of very low amplitude<sup>2</sup>. As the oxygen partial pressure ( $p_{O_2}$ ) of arterial blood decreases eventually to 5 mmHg and beyond, the animals remain quiescent for longer and longer periods between bouts of activity. At these times, a most unusual,

but nevertheless repeatable, observation is the occasional reversal of the normal arterial–venous blood  $p_{O_2}$  gradient, when venous  $p_{O_2}$  actually exceeds that of the arterial blood (Fig. 2a, b). Calculation of the corresponding cardiac output by the Fick equation at this time obviously gives the embarrassing result that blood is flowing in reverse. We are reasonably certain this is not the case, and instead we believe that blood flow is so slow and intermittent during severe hypoxia that the vena cava, which is positioned superficially in the roof of the mantle cavity and is of considerable volume, has time to equilibrate with the ambient  $p_{O_2}$ . At the same time, the arterial cannula in the heart picks up blood that has been stationary in the ventricle for some time (although there is still a heart rate, the times between beats can exceed the times during which blood samples might be taken). When ventilatory movements have all but ceased, it therefore seems that the vena cava becomes in effect a functional gill, scavenging what little  $O_2$  remains available, in a most energy-efficient manner.

The extent to which *Nautilus* can exploit the oxygen stores in the blood as well as in the buoyancy chambers of the shell is disputed<sup>2,5–7</sup>. The  $O_2$  store in the shell chambers of an animal resting at 18 °C are estimated at 6.9 ml for a 450-g (flesh weight) *Nautilus*, with an available shell-gas volume of 100 ml (ref. 2), and a shell-gas  $p_{O_2}$  of 54.6 mmHg (Fig. 3). The same animal, with a blood volume of 90 ml (ref. 2) equally distributed between a 30%  $O_2$ -saturated venous and 70%  $O_2$ -saturated arterial compartment, and a maximal blood-oxygen-carrying capacity of 2.3 ml  $O_2$  per 100 ml (ref. 8), would have 1.0 ml  $O_2$  available in the blood. With this available blood-oxygen store, a 450-g *Nautilus*, with a resting  $\dot{V}O_2$  (oxygen consumption) of 0.28 ml  $O_2$  per min (Fig. 1), could either descend into an anoxic zone or retreat into its shell, and have enough oxygen to sustain aerobic metabolism for ~3.5 min. The impact of lowering its metabolic rate to the levels seen during severe hypoxia (Fig. 1) would be to extend this time to ~50 min. However, if the rates of  $O_2$  transfer between shell gas and blood were high enough to support the metabolic rate, this could extend the time by up to 6 h. An upper estimate of the rate that oxygen might feasibly be metered from the shell can be made by assuming that  $O_2$  would diffuse through the wall of the siphuncle as rapidly as through water. The volume of oxygen that could so diffuse can be calculated by the equation,  $\Delta V_{O_2} = -DA(\Delta x/\Delta y)\Delta \text{time}$ , where  $D$  is the diffusion coefficient for  $O_2$  in water ( $25 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ),  $A$  is the total siphuncular surface area ( $13 \text{ cm}^2$ , ref. 5) and  $\Delta x/\Delta y$  is the concentration gradient between shell gas and blood,  $\Delta y$  being the diffusion barrier thickness (0.027 cm; ref. 5). At the 31‰ salt concentration of cameral

FIG. 2 a, Ventilation rate and cardiac output measured at the time of blood sample withdrawal. b, Arterial and venous  $p_{O_2}$  measurements. Data are from an individual animal undergoing progressive hypoxia in a closed respirometer, as in Fig. 1. Chronic indwelling catheters were implanted 24 h before the experiments to enable repetitive sampling of arterial and mixed venous blood from undisturbed animals as before<sup>2</sup>. c, Simultaneously recorded arterial and venous  $p_{O_2}$  levels for 6 animals undergoing progressive hypoxia as in Fig. 1. In the latter stages of the experiment, when hypoxia was most severe, ventilation rate was highly intermittent in all animals. In four of the six animals studied, repetitive blood samples taken to coincide with the periods before and after the most prolonged of these respiratory pauses, revealed that the venous  $p_{O_2}$  occasionally exceeded that of simultaneously drawn samples of arterial blood (note the reversed arterial–venous  $p_{O_2}$  gradients).  $p_{O_2}$  measurements were made on blood samples drawn in Hamilton gas-tight syringes using Radiometer E-5046 electrodes and D616 cuvettes thermostatted to 18 °C. Electrodes were preconditioned before every measurement to the approximate blood gas levels as detailed earlier<sup>19</sup>, and calibrated with precision gas mixtures before and after each measurement, with linear interpolation being used to account for any small drift.



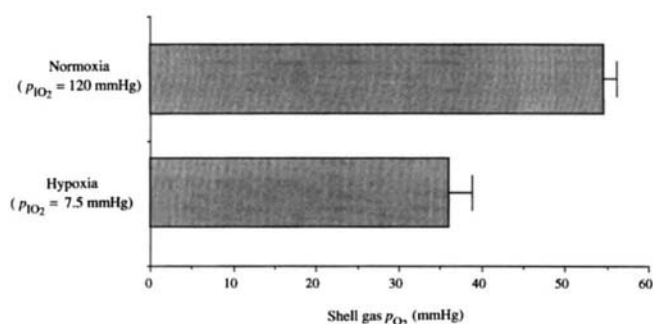


FIG. 3 Partial pressures of oxygen  $P_{O_2}$  in cameral gas samples taken from 5 normoxic animals and in the same animals after they had been subjected to 8 h of hypoxia, as in Fig. 1. The day before an experiment, gas sampling ports were drilled into two of the most recently formed shell chambers. Gas-tight chromatography septa were then immediately sealed in place over each hole using cyanoacrylate glue. Gas samples of 0.5 ml were taken with Hamilton gas-tight syringes and measured with Radiometer electrodes and cuvettes as in Fig. 2. Measurements of cameral gas  $p_{O_2}$  in normoxic animals made 12–16 h before the experiments began were not significantly different (Student's paired  $t$ -test,  $P < 0.05$ ) from the samples taken immediately before the exposure of animals to hypoxia, so we can be reasonably certain that the decline in  $p_{O_2}$  during 8-h hypoxia was due directly to the environmental  $O_2$  lack, and not to a time-dependent fall in  $p_{O_2}$  in normoxia.  $p_{IO_2}$ , inspired  $P_{O_2}$ .

fluid<sup>5</sup> and a shell  $p_{O_2}$  of 54.6 mmHg (Fig. 3), the cameral fluid would contain  $(54.6/155) \times 5.98 \times 10^{-3} = 2.107 \times 10^{-3}$  ml  $O_2$  per ml fluid. Therefore, the  $\Delta V_{O_2}$  (ml) =  $25 \times 10^{-6} \times 13 \times (2.107 \times 10^{-3}/0.027) \times 3600 = 0.09$  ml  $O_2$  h<sup>-1</sup> for a 450-g *Nautilus*. At the measured hypoxic metabolic rate of 1.19 ml  $O_2$  h<sup>-1</sup>, the fraction attributed to the shell  $O_2$  would be ~8%. On the other hand, the data in Fig. 3 reveal a mean  $p_{O_2}$  decline in the shell gas of 18.5 mmHg (2.4 ml  $O_2$ ) over 8 h, which is equivalent to a transfer rate of 0.3 ml  $O_2$  per h, and to a fractional contribution (0.3/1.19) of 25%. Even greater contributions may be possible at *Nautilus* deep-water habitat temperatures of 8–9 °C (ref. 7) where, with a  $Q_{10}$  approximating 3–4 (refs 9,10), the shell  $O_2$  store could sustain a large proportion of the animal's hypoxic metabolic rate.

*Nautilus* is unique amongst the cephalopod molluscs in having the capacity to survive prolonged periods of low  $O_2$  levels. It

accomplishes this by sharing the mechanisms used by other 'good' facultative animal anaerobes<sup>3,4</sup>, namely a marked aerobic metabolic rate suppression and a modest reliance on anaerobic energy production. However, the hypometabolic, hypoxic *Nautilus* may well live up to its reputation as a zoological oddity, as much in function as in form, if we are correct in suggesting the ability to use a superficial circulation to load its blood with  $O_2$  at the venous side and to utilize, albeit slowly, the oxygen contained in its buoyancy chambers to help sustain aerobic metabolism. To speculate whether a similar suite of hypoxic defence mechanisms contributed to the success of ectocochleate forms, by enabling them to exploit the  $O_2$ -deficient waters of the Palaeozoic for refuge or feeding<sup>2,11,12,13</sup>, adds to the debate over which features were to remain shared after the early divergence of the nautiloid and ammonoid lineages<sup>14</sup>. □

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## A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*

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A PHEROMONE-INDUCED neurosecretory pathway in *Caenorhabditis elegans* triggers developmental arrest and an increase in longevity at the dauer diapause stage. The gene *age-1* is required for non-dauer development and normal senescence. *age-1* encodes a homologue of mammalian phosphatidylinositol-3-OH kinase (PI(3)K) catalytic subunits. Lack of both maternal and zygotic *age-1* activity causes dauer formation, whereas animals with maternal but not zygotic *age-1* activity develop as non-daughters that live more than twice as long as normal. These data suggest that phosphatidylinositol signalling mediated by AGE-1 protein controls lifespan and the dauer diapause decision.

TABLE 1 Concentrations of anaerobic metabolic end-products in normoxic and hypoxic *Nautilus*

|          | Adductor muscle |           |
|----------|-----------------|-----------|
|          | Octopine        | Succinate |
| Normoxia | 0.6 ± 0.1       | 1.2 ± 0.2 |
| Hypoxia  | 7.2 ± 1.3       | 1.8 ± 0.4 |

Adductor muscle was taken from freshly killed animals and rapidly frozen in liquid nitrogen for subsequent analysis of anaerobic end-products as before<sup>15</sup>. Values are means ± s.e.m. ( $n = 6$ ) for both normoxic animals at rest and for those sampled after 500 min progressive hypoxia. Octopine and succinate concentrations are given in  $\mu$ mol per g wet weight. In molluscs, the terminal dehydrogenase of anaerobic glycolysis is that of octopine<sup>16</sup> which is formally analogous to lactate dehydrogenase in vertebrates<sup>17</sup>. Succinate represents another alternative anaerobic fermentation pathway in many molluscs<sup>17,18</sup>. Estimates of ATP turnover, calculated from normoxic and hypoxic aerobic metabolic rates (168 and 11.9  $\mu$ mol per kg ATP per min, respectively) and from the accumulation of octopine and succinate<sup>17,18</sup> in hypoxic animals (7.1  $\mu$ mol per kg ATP per min) indicate that accelerated glycolytic flux makes up only 4.5% of the  $O_2$  deficit. Anaerobic ATP turnover was calculated assuming that the metabolite accumulation in adductor muscle reflected that of the total muscle mass of a 1-kg animal, having a shell weight of 333 g and a muscle mass of 230 g (40% of flesh weight).

TABLE 1 Regulation of lifespan and dauer formation by *age-1*

| Paternal genotype           | Maternal genotype           | Zygotic genotype            | Age phenotype    | Lifespan (days) | Lifespan relative to wild type |
|-----------------------------|-----------------------------|-----------------------------|------------------|-----------------|--------------------------------|
|                             | +/+                         | +/+                         | non-dauer (1000) | 8.1 ± 0.2 (90)  | 1.0                            |
|                             | <i>mg44</i> /+              | <i>mg44</i> / <i>mg44</i>   | non-dauer (505)* | 20.7 ± 0.2 (26) | 2.6                            |
|                             | <i>mg44</i> / <i>mg44</i>   | <i>mg44</i> / <i>mg44</i>   | dauer (505)      |                 |                                |
|                             | <i>hx546</i> / <i>hx546</i> | <i>hx546</i> / <i>hx546</i> | non-dauer (1000) | 16.6 ± 1.0 (43) | 2.0                            |
| +/+                         | <i>mg44</i> / <i>mg44</i>   | <i>mg44</i> /+              | non-dauer (100)  | 10.7 ± 0.5 (35) | 1.3                            |
| <i>hx546</i> / <i>hx546</i> | <i>mg44</i> / <i>mg44</i>   | <i>hx546</i> / <i>mg44</i>  | non-dauer (100)  | 19.8 ± 1.1 (54) | 2.4                            |
|                             | <i>mg44</i> / <i>hx546</i>  | <i>mg44</i> / <i>mg44</i>   | dauer (406)      |                 |                                |

The *age-1*(*mg44*) chromosome is marked in *cis* with *sqt-1*(*sc13*), and *age-1*(*mg44*)/+ strains have the *mnC1* balances chromosome as the + chromosome. Lifespan at 25 °C is measured from the L4 stage to the last day that animals respond to light touch, and is presented as the mean ± s.e. Note that *sqt-1*(*sc13*) *age-1*(*mg44*) progeny of *age-1*(*hx546*)/*sqt-1*(*sc13*) *age-1*(*mg44*) form dauer larvae with 100% penetrance at 25 °C. This is the same as control *age-1*(*mg44*) daughters of *age-1*(*mg44*) parents. The dauer larvae produced in both cases have the dark intestine, pharyngeal and cuticular remodelling, and arrest of the moulting cycle characteristic of this stage<sup>3,5</sup>. In contrast, 99% of *sqt-1*(*sc13*) *age-1*(*mg44*) progeny of *sqt-1*(*sc13*) *age-1*(*mg44*)/+ do not form dauer larvae, showing that maternal *age-1* activity is sufficient to allow non-dauer development. However, these animals live 2.6-fold longer than wild type. Zygotic *age-1*(+) in the absence of maternal *age-1* supplies sufficient activity for normal lifespan and non-dauer development, but zygotic *age-1*(*hx546*) does not supply sufficient activity for normal longevity. Similar results were observed with other *age-1* alleles *m333* and *mg109* (data not shown). We three-factor mapped *age-1*(*hx546*) to the 1.2 map unit *sqt-1* *lin-29* interval. Of 20 recombinants in this interval, 4 were between *sqt-1* and *age-1*(*hx546*), and 16 were between *age-1* and *lin-29*. This shows that *age-1*(*hx546*) maps to the same genetic interval as *age-1*(*mg44*)<sup>5</sup>. The *age-1*(*hx546*) lifespan and failure maternally to rescue *mg44* phenotypes co-segregated in the 13 recombinants tested for both phenotypes (2/13 *age-1*(*hx546*) and 11/13 *age-1*(+)). Wild-type or *age-1*(*hx546*) males were mated into *sqt-1*(*sc13*) *age-1*(*mg44*) hermaphrodites. Cross progeny were picked to separate plates at 25 °C and F<sub>2</sub> progeny were scored 3 days later. For genetic mapping, recombinants were picked from *age-1*(*hx546*)/*sqt-1*(*sc13*) *lin-29*(*n333*) hermaphrodites. Recombinants were tested for failure to maternally complement *age-1*(*m333*) for dauer formation and lifespan at 25 °C.

\* 1% of these worms form dauer larvae, and 20% become sterile adults.

Mutations affecting dauer formation (*daf* mutations) have been ordered into a genetic epistasis pathway that is likely to represent the steps in the development or function of a neuroendocrine system<sup>1-6</sup>. Both longevity and dauer formation are regulated by *daf-23*: maternally rescued *daf-23* mutants have dramatically increased longevity, whereas non-maternally rescued *daf-23* mutants form dauers constitutively<sup>7,8</sup>. A genetic screen for increased longevity identified *age-1*(*hx546*) (refs 9, 10), which forms dauers constitutively at high temperature (27 °C), and fails to complement *daf-23* (T. Inoue and J. Thomas, personal

communication). We find that under more standard conditions (25 °C), *age-1*(*hx546*) fails to supply maternal *daf-23* gene activity, and *daf-23*(*mg44*) fails to supply zygotic *age-1* activity (Table 1). In addition, *age-1*(*hx546*) maps to the same genetic interval as *daf-23* (Table 1). We therefore assign to *age-1* both *hx546* and the dauer constitutive alleles (*mg44*, *mg55*, *m333* and *mg109*) previously assigned to *daf-23* (refs 5, 8). As shown below, the maternal effect dauer arrest of *mg44* is the null phenotype, whereas the long lifespan of *hx546* is a reduction-of-function phenotype.

We mapped *age-1* to a 700-kilobase region covered by 4 contigs.

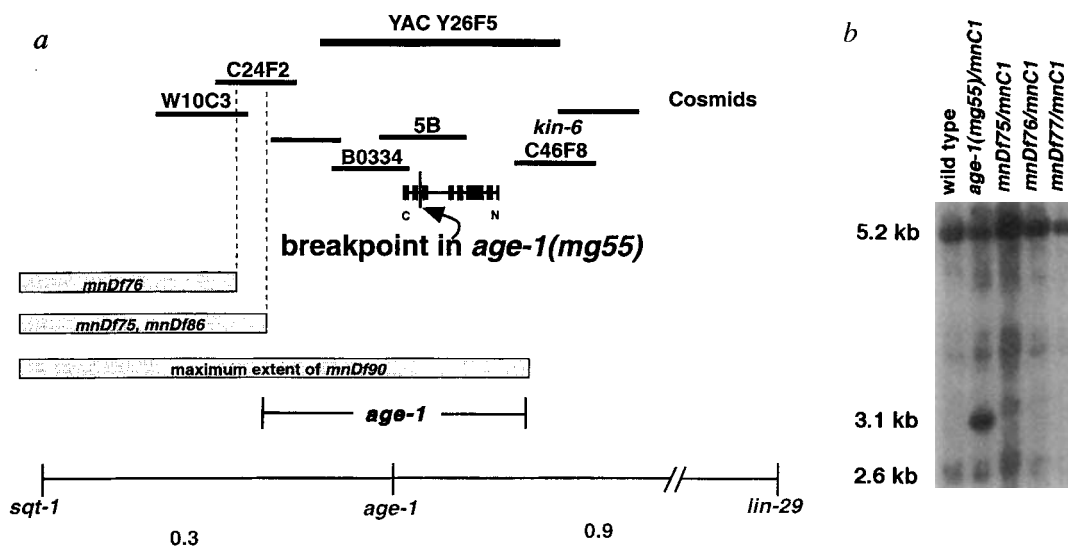


FIG. 1 a, Physical genetic map of the *age-1* region, with *age-1* transcribed from right to left. Cosmid and yeast artificial chromosome (YAC) clones were placed by the *C. elegans* Genome Project. *mnDf75*, *mnDf76* and *mnDf86* all complement *age-1* and fail to complement *sqt-1*. Cosmid C24F2 detects breakpoints on Southern blots in all three deficiencies. W10C3 partly overlaps C24F2 and detects a breakpoint in *mnDf76* but not in *mnDf75* or *mnDf86*. Thus *age-1* must be located right of the breakpoints in C24F2. PCR performed on embryos homozygous for *mnDf90*, which fails to complement both *sqt-1* and *age-1*, shows that this deficiency deletes *sqt-1* but not *kin-6*, so *age-1* is to the left of *kin-6*. (The *age-1* transcript and phage 5B are not drawn to scale). b, Rearrangement breakpoint in

*age-1*. Southern blot showing hybridization of *Hind*III-digested genomic DNA isolated from wild-type (N2) (lane 1), *age-1*(*mg55*)/*mnC1* (lane 2), and three *Df*/mnC1 control strains (lanes 3–4, 5) to a probe made from a 4-kb *Sal*I subclone of phage 5B. This probe detects the region that encodes the C-terminal region of AGE-1. The strain bearing *mg55* shows both a 5.2-kb *Hind*III fragment from the wild-type *age-1* allele on *mnC1* and an altered 3.1-kb *Hind*III fragment from the *age-1*(*mg55*) allele. METHODS. PCR was performed on single arrested L1 larvae (*mnDf75*, *mnDf76*, *mnDf86*) or single dead eggs (*mnDf90*) laid by heterozygous deficiency/mnC1 animals, using standard techniques.

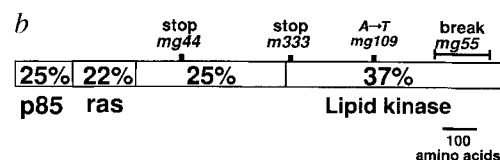
**a**

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1  MHVNILHPQLQTMVEQWQMRERPSLETENGKSGLLLENAGVADIIITMCPFF
51  GEVISVVPFWLANVRTSLKLSDFKHQFLFELIAPMKWGTYSVKPQDVV
101  FRQLNNFGIEIVFNDDQQLSKLELHGTFFMFLYQPDGINRDKELMSDI
151  SHCLGSLDKLEESLDEELRQFRASLWARTKKTCLTRGLEGTSHYAFPE
201  QYLCVGESCPKDLSEKVKAAKLSYQMFWRKRKAELNGVCEKMMKIQTFFN
251  PNETPKSLHTFLSRNAIKLDVYDTPDDEGWFERSLAGRTTEVTNP
301  VKLTSYDVRLQONIFVQLKLNFGQVRSERRRLCPGFVVRQSLVLKDYCR
351  PKPLYEPHYVRAHERKLALDLVLSVSDSTPKQSKNSDMVMTDFRPTASLK
401  QVSLDOLDANLMIRPVNISGDFPADVDMYVRFEFSVYVGLTLTASKST
451  KVNAQFAKWNKMYTFDLYMKDMPFSAVLISIRVLYGKVKLSEFEFVGWV
501  NMSLTDWRDELRCQGFLLHFWAEPETANRSRIGENGARIGTNAAVTIEIS
551  SYGGRVRMPSQOQYTYLVKHRSTWTETLIMGDDYESCIRDPGYKKLQML
601  VKKHESGIVLEDEQRHVMWRRYIQKQEPDLLIVLSELAFVWTDRENF
651  ELYVMLEKWKPPSVAAALTLGKRCRDRVIRKFAVEKLNQLSPVTFHLF
701  ILPLIQAQKYEPRAQSEVGMMLLTRALCDYRIGHRLFWLLRAEIALRLDC
751  DLKSEBYRRTISLMEAYLRGNEEHKIIIRQVDMVDELTRISTLVKGMPK
801  DVATMKLRDELRSISHKMENMDSPLDVPVYKLGEMIIDKAVLGSARKPLM
851  LHWKKNKPKSDLLHLPFCAMIFKNGDDLRLQDMLVLQVLEVMNDIWKANID
901  CCLNPAVALPMGEMTGIIEVVPNCKTIFEIQVGTGFMTAVRSIDPSEFMN
951  KWIRKQCGIEDKKKSKKDSTKNPIEKKLDNTQAMKKYFESVDRFLYSVC
1001 GYSVATYIMGIKDRHSDNLMLTEDGKYFHIDFGHILGHGKTKLGICDRQ
1051 PFILTEHFMVTRSGKSDVSDGNSHELQKFKTLCVEAYEVMMNNRDLFVSLF
1101 TLMGLMELPELSTKADLDHLKKTLCFNGESKEEARKFFAGIYEAFNGSW
1151 STKTNWLFHAVKH

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FIG. 2 **a**, The AGE-1 protein sequence. Amino acids in bold are identical between AGE-1 (accession number U56101) and mouse p110 $\alpha$  (accession number P42337). The amino acids altered in the *mg44*, *m333* and *mg109* lesions are underlined. The *mg55* breakpoint is not shown, but was mapped to lie between amino acids 996 and 1,111. **b**, AGE-1



lineup with mouse p110 $\alpha$ . The diagram is to scale, and extends over the 1,164-amino-acid AGE-1 sequence. Percentage identities shared between each domain of mouse p110 $\alpha$ <sup>18,30</sup> and the region of AGE-1 aligned with it by the Gap are given. The domains labelled ras and p85 have been shown in p110 $\alpha$  to bind ras and p85, respectively. The lipid kinase domain includes the region of AGE-1 that shares extensive homology with all lipid kinases. The approximate location of each point mutation is shown relative to the putative AGE-1 domains predicted by Pileup.

**METHODS.** Sequence of *age-1* was determined by sequencing cDNA clones, amplified reverse-transcribed PCR fragments, and genomic regions described in Fig. 1. The sequence is likely to be complete because the 5' ends of 3 independent cDNAs map within 30 bases of each other, and because it encodes a protein coextensive with other PI(3)K p110 subunits. PCR-generated templates were sequenced without subcloning and verified by sequencing multiple isolates. The *age-1* alleles were sequenced by PCR amplifying genomic regions and direct sequencing of templates without subcloning. Each mutation detected was verified by sequencing an independently amplified template. Percentage identities were calculated by the Gap program.

Analysis of a set of chromosomal deficiencies in the *age-1* region delimited the *age-1* location to a 240-kb interval (Fig. 1a). B0334, a cosmid from this interval that was sequenced by the *C. elegans* Genome Project<sup>11</sup>, detects a breakpoint associated with the *age-1(mg55)*  $\gamma$ -ray-induced allele (Fig. 1b). This breakpoint disrupts an open reading frame that extends off the right end of B0334. We isolated genomic and cDNA clones extending to the right from B0334, and used reverse transcription-polymerase chain reaction (RT-PCR) to determine the sequence of the coding region of *age-1* in wild-type and *age-1* mutants. Four *age-1* mutations were detected in this open reading frame (see below).

The *age-1* cDNA sequence predicts a protein of 1,164 amino acids, that is most similar to mammalian PI(3)K p110 catalytic subunits (Fig. 2). Blast analysis indicates that AGE-1 is much more closely related to PI(3)K (random probability of alignment:  $e^{-113}$  for p110 $\alpha$ ,  $e^{-101}$  for p110 $\beta$ , and  $e^{-93}$  for p110 $\gamma$ ) than to PI(4)K ( $e^{-22}$ ) or DNA repair kinases ( $e^{-8}$ ) that have also been classified in this kinase superfamily<sup>12,13</sup>. Three classes of PI(3)K p110 subunits generate a membrane-localized signalling molecule, phosphatidylinositol-3,4,5-trisphosphate (PtdInsP<sub>3</sub>), which is thought to transduce signals from upstream receptors to effector molecules such as the AKT/PKB kinase<sup>12,14,15</sup>. The p110 $\alpha$  lipid kinase is activated by regulatory p85 or p55 subunits that bind to phosphorylated tyrosines on receptor and non-receptor kinases<sup>12,16,17</sup>. PI(3)K may also signal by a protein kinase cascade because p110 $\alpha$  phosphorylates p85 as well as lipids<sup>17,18</sup>. p110 $\gamma$  is regulated by heterotrimeric G proteins<sup>19</sup>. AGE-1 may couple to a p85-like tyrosine kinase adaptor, rather than to a heterotrimeric G protein. In the 130-amino-acid N-terminal region that in p110 $\alpha$  couples to p85 (ref. 18), AGE-1 is more related to p110 $\alpha$  (22.0% identity) than to p110 $\beta$  (17.1% identity) or p110 $\gamma$  (9.8% identity). However, AGE-1 is less similar to the mammalian PI(3)K types than they are to each other, so AGE-1 may define a divergent PI(3)K class (Fig. 2).

Analysis of *age-1* mutant alleles shows that the AGE-1 PI(3)K functions specifically in the dauer arrest and longevity pathway. The *age-1(mg44)* mutation (Trp405Amber) and the *age-1(m333)* mutation (Trp659Opal) are predicted to truncate AGE-1 upstream of the kinase domain and other conserved regions and produce no *age-1* activity. The *age-1(mg55)* breakpoint disrupts the kinase domain. The *age-1(mg109)* Ala845Thr substitution alters a conserved protein region (Fig. 2). The *mg44* and *m333* null alleles cause similar zygotic longevity and maternal-effect, dauer-arrest phenotypes<sup>5,8</sup> (Table 1). Animals with no maternal or

zygotic *age-1* activity (*age-1(mg44)* progeny of *age-1(mg44)* parents) arrest development as dauer larvae<sup>5</sup> (Table 1). The contribution of only maternal *age-1* activity (*age-1(mg44)* progeny of *age-1(mg44)/+* parents) is sufficient for non-dauer development, but the lifespan of these animals increases by two- to threefold<sup>8</sup> (Table 1). In the absence of maternal *age-1* contribution (*age-1(mg44)/+* progeny of *age-1(mg44)* mothers), *age-1* zygotic activity is sufficient to allow non-dauer development and nearly normal lifespan (Table 1). These data suggest that normal senescence depends on phosphatidylinositol signalling from zygotic AGE-1, whereas either maternal or zygotic *age-1* activity is sufficient to allow non-dauer development.

Based on precedents from vertebrates, we propose that AGE-1 PI(3)K could function in the development<sup>20,21</sup> or neuroendocrine signalling<sup>22,23</sup> of the dauer pathway. Whereas absence of AGE-1 activity induces many dauer characteristics, reduced AGE-1 PI(3)K activity may trigger a subset of the dauer program that includes a decrease in the rate of senescence. Analogous neuroendocrine pathways mediate senescence in mammals<sup>24</sup>. Free radicals have been implicated in senescence<sup>24</sup>, and *age-1* mutants and dauer larvae are more resistant to free radicals and express higher levels of catalase and superoxide dismutase than wild-type non-daughters, suggesting that AGE-1-mediated signalling normally inhibits a free-radical protection pathway<sup>25,26</sup>. PI(3)K has also been implicated in the production of superoxide radicals by mammalian neutrophils<sup>27,28</sup>.

Genetic data indicate that *daf-16* could be a negatively regulated target of PtdInsP<sub>3</sub> signalling. Both the longevity and dauer-arrest *age-1* phenotypes are suppressed by *daf-16* (refs 5, 8, 29). These observations suggest that during normal senescence the PtdIns<sub>3</sub> product of AGE-1 negatively regulates DAF-16 activity to control lifespan in *C. elegans*. It is not yet known whether phosphatidylinositol signalling in longevity control is peculiar to *C. elegans* or other species that enter diapause, or whether it is phylogenetically more general. □

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## Attentional modulation of visual motion processing in cortical areas MT and MST

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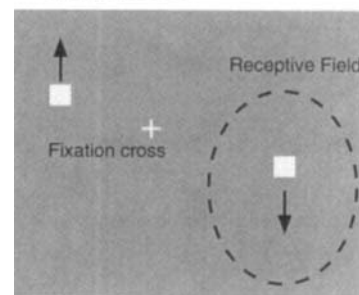
THE visual system is constantly inundated with information received by the eyes, only a fraction of which seems to reach visual awareness. This selection process is one of the functions ascribed to visual attention<sup>1–6</sup>. Although many studies have investigated the role of attention in shaping neuronal representations in the visual cortex, few have focused on attentional modulation of neuronal signals related to visual motion. Here we report that the responses of direction-selective neurons in monkey visual cortex are greatly influenced by attention, and that this modulation occurs as early in the cortical hierarchy as the level of the middle temporal visual area (MT). Our finding demonstrates a stronger and earlier influence of attention on motion processing along the dorsal visual pathway than previously recognized.

Using standard extracellular techniques, we recorded from neurons in MT and the medial superior temporal area (MST) in the superior temporal sulcus of two behaving macaque monkeys. Both areas contain a high proportion of direction-selective cells<sup>7–9</sup>, and their sensory response to moving stimuli has been extensively studied<sup>10</sup>. The animals were trained in a task that allowed us to compare the responses of individual neurons to identical visual stimuli under different attentional conditions. By comparing neural responses only between conditions of identical visual stimulation, and by strictly monitoring fixation with a scleral search coil, we ensured that the differences in neural response between the various attentional conditions were due solely to changes in the behavioural state of the animal.

The stimuli consisted of small bright dots presented on an otherwise dark computer monitor in front of the animal. Each

trial began with the presentation of a small fixation cross on the screen (Fig. 1). After the monkey had fixated this cross, a stationary dot appeared somewhere on the screen, generally a few degrees to the left or right of the fixation point. The animal responded by depressing a lever which caused one (experiment 1) or two (experiment 2) other dots to appear. All dots immediately started to move back and forth along straight, non-crossing paths at the same speed (but not necessarily in the same direction). The animal's task was to track the dot that had appeared first (the 'target') (attentionally, rather than with the eyes) and to release the lever quickly when this dot changed speed. The other dots ('distractors') might also change speed, but the trial was terminated without reward if the animal responded to a speed change of

a Experiment 1



b Experiment 2

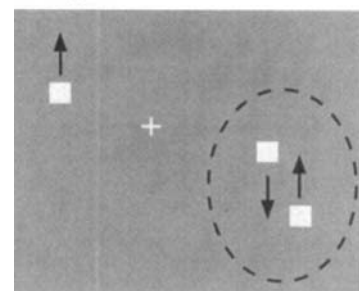


FIG. 1 Stimulus conditions for experiments 1 (a) and 2 (b). The dashed line is the circumference of the receptive field, plotted by hand using a freely movable dot or light bar while the animal fixated a small spot. The cross marks the fixation spot. a, One dot moved through the receptive field along the cell's preferred and null directions while the other dot moved (not necessarily parallel to the first dot) outside the receptive field. b, A further dot was added inside the receptive field, moving parallel to but in the opposite direction to the other dot. All dots (~0.5 × 0.5°) travelled along straight paths at a constant speed (roughly matched to a cell's preferred speed) and their directions were reversed at the same time. The animal was instructed which dot to attend to by presenting it alone and stationary at the beginning of the trial. The animal had to depress the lever at this point which would make the other dot(s) appear and all dots would immediately start moving. The magnitude of the speed change was varied between cells roughly to match the performance of the animal for the given receptive field location, size and preferred speed. In experiment 1 the speed increases were about 30–55%. Excluding the trials that were aborted because of an eye movement the average rate of correct responses was 90% (5% target speed change missed; 5% responses to distractor dot or unknown reason). In experiment 2, the animal achieved about 70% correct responses even with speed increases of 40–70% (14% target speed change missed, 10% responses to speed change in a distractor dot, 6% unknown reason). Unless we lost the cell early the number of correct trials per trial type was about 10–20, although the total number varied. Motion trajectories were roughly matched to the size of the classical receptive field, except for the small receptive fields of the MT cells with small eccentricity. The separation of the two paths inside the receptive field in experiment 2 was generally about 0.5 to 2°. Eye positions were analysed to ensure that differences in neuronal responses could not be attributed to fixation differences. The median difference in fixation position between trial types was less than 0.15° for both experiments (receptive fields were rarely less than 6° across).

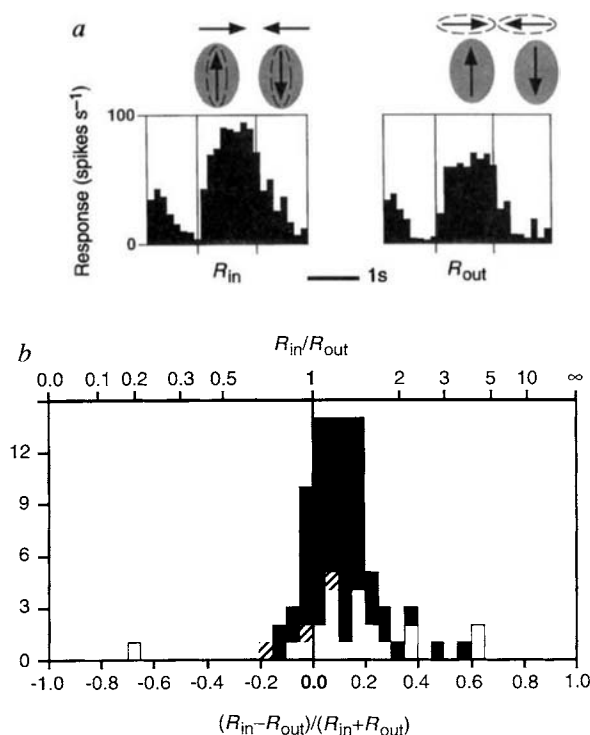


FIG. 2 Effects of attention on responses in experiment 1. *a*, Responses of an isolated neuron in MT, when attending either to the dot inside (left) or outside the receptive field (right). Stimulus motion is shown above, with the attended stimulus encircled by a dashed line and the shaded area symbolizing the receptive field. Vertical lines on the histograms mark the times when the dots reversed direction. The response from the second period, when the dot inside the receptive field was moving in the neuron's preferred direction, was used for the analysis. For this cell the response was about 30% stronger when the receptive-field stimulus was the target. *b*, Stacked histogram showing the strength of attentional modulation for all neurons tested in MT (black bars: 46 cells from animal S and 19 cells from animal D) and MST (grey bars: animal S, 6 cells; animal D, 15 cells) and for the three cells that were either in MT or MST (white bars: all from animal S). An attentional index was computed:  $AI = (R_{in} - R_{out}) / (R_{in} + R_{out})$ , where  $R_{in}$  is the response to the preferred motion inside the receptive field when the target dot is the stimulus inside the receptive field and  $R_{out}$  is the response to the same visual stimulation when the target is the dot outside the receptive field. The upper x-axis shows corresponding ratios of responses ( $R_{in}/R_{out}$ ). Testing the cells individually with a two-tailed *t*-test only 4 (24%) of the negative indices were significantly different from zero ( $P = 0.05$ ) whereas 44 (61%) of the positive indices were significantly larger than zero. The median modulation was 19% for MT cells and 40% for MST cells. Because the index was on average larger for cells from animal D we tested for the inter-area difference for significance separately for the two animals (two-tailed *t*-test). The difference was significant in animal D. Because of the small number of MST cells from animal S, the difference did not reach significance.

a distractor. Throughout the trial, the animal had to maintain its gaze on the fixation cross. Only those portions of correctly completed trials, before any dot had changed speed, were analysed.

We recorded from 96 direction-selective neurons in the superior temporal sulcus. Histological reconstruction from myelin-stained sections showed that 65 of these cells were in MT, 21 in the lateral or dorsal subdivisions of MST, and 3 in either MT or MST. The remaining 7 cells were excluded from the analysis as they were near the MT/V4 border and could not be assigned to MT with certainty.

When a neuron was isolated, one (experiment 1) or two (experiment 2) dots were positioned to move back-and-forth within its receptive field, with their axis of motion aligned to the cell's preferred direction. Experiment 1 was designed to test the effect of directing attention either inside or outside the receptive field of the cell, while maintaining identical visual stimulation. Figure 2*a* shows the response of a neuron in MT to the back-and-forth motion of the dot within its receptive field, under these two conditions. The left panel is a histogram of the cell's response during trials where the animal was instructed to attend to the dot inside the receptive field (with the distractor outside), and the right panel shows the response when the target was the dot outside the receptive field (with the distractor inside). The visual stimulation was thus kept identical. Like most cells we encountered, this neuron responded more strongly when the stimulus inside its receptive field was the target. The median value for this enhancement was 19% for cells in MT, and 40% for cells in MST. The strength of attentional modulation for all sampled MT and MST cells is summarized in Fig. 2*b*.

In the second experiment an additional dot was presented inside the receptive field, moving parallel to the other dot, but always in the opposite direction. On a given trial, any one of the three dots could be the target. The responses of most neurons depended greatly on which of the dots was the target. The responses of one MT cell are shown in Fig. 3*a*. When the animal was instructed to attend to either of the dots in the receptive field, the neuron responded most strongly when that dot moved in the cell's preferred direction (upwards). When the other dot in the receptive field was the target, the phase of the response changed,

so that the neuron now responded most strongly when that other dot was moving in the preferred direction. Thus, the neuron encoded the movement of the target, even if a more powerful sensory stimulus was present in the receptive field. When the animal was cued to attend to the dot outside the receptive field, the neuron maintained a relatively steady level of activity, between the level of responses to the preferred and null motion direction alone, as observed in the first experiment, when the animal attended to the dot outside the receptive field. This intermediate level of activity reflects the previously observed response suppression in MT using transparent stimuli<sup>11</sup>. When the target moved in the null direction inside the receptive field the response of the neuron was depressed below that evoked when the target was outside the receptive field.

We quantified the strength of the attentional modulation in experiment 2 by comparing for each neuron the response during the second phase of motion, while one or the other receptive field dot was the target. The index distributions in Fig. 3*b* show that almost all MT and MST neurons responded most strongly when the attended dot was travelling in the preferred direction. The median enhancement was 86% for MT and 113% for MST, that is, the neural response was roughly doubled when the stimulus moving in the preferred direction was the target dot.

These results demonstrate a powerful effect of attention on the processing of visual motion information. The responses of neurons in MT and MST are reduced when attention is directed to a stimulus outside their receptive fields. When one of two dots moving inside the receptive field is the target, the responses of the cells depend primarily on the movement of that stimulus. The influence of the distractor dot is much reduced, even if it is a more powerful sensory stimulus. Earlier reports have described extraretinal effects in areas in the dorsal pathway beyond MT (MST, Areas 7 and 7a (refs 12–16); and in positron emission tomography (PET) studies of human parietal cortex<sup>17,18</sup>, but previous single-unit studies failed to find evidence for appreciable systematic extraretinal effects in MT<sup>14,19,20</sup>. In contrast, we found robust attentional effects in most of the neurons we encountered in this area. It is likely that this difference is due to differences in the tasks employed. In particular, our first experiment, which uses a design similar to many previous studies, shows a much smaller

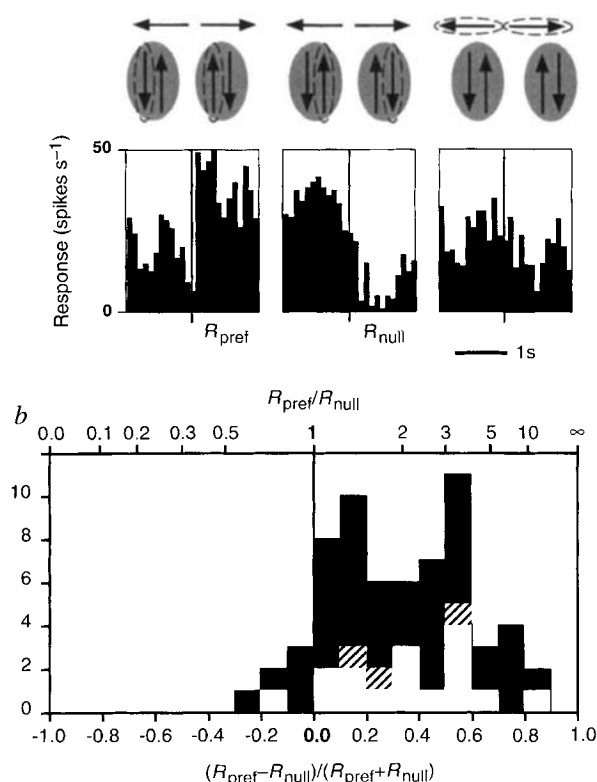


FIG. 3 Responses with two dots inside the receptive field. *a*, Responses of a neuron in MT during experiment 2, when three dots were presented. The left and central histograms show responses when the animal had been instructed to attend to either of the two dots in the receptive field, the right histogram plots responses when the target was the dot outside the receptive field. The axis of motion of the dot outside the receptive field relative to the axis of motion of the dots inside varied from cell to cell. When the target dot was inside the receptive field, the response of the neuron was strong whenever that dot (circled) moved in the preferred direction. The activity was relatively unmodulated at an intermediate level when the animal was attending to the dot outside the receptive field (shown for reference only, and not used for analysis). *b*, Stack histogram of the attention index for the subset of cells (44 MT cells; 16 MST cells) from experiment 2 (labels as in Fig. 2). Each index is computed using the average rate of firing, when the target dot was moving in the preferred direction (marked  $R_{pref}$ ) inside the receptive field, compared with the response when the animal was attending to the dot moving in the null direction (marked  $R_{null}$ ) inside the receptive field. The median modulation was 86% for MT cells and 113% for MST cells. This difference in modulation was significantly different between these two cell types in animal D, while it was not significant in animals because of the small MST sample. Testing the cells individually with a two-tailed *t*-test, only 1 (17%) of the negative indices was significantly different from zero  $P < 0.05$  whereas 47 (82%) of the positive indices were significantly larger than zero.

attentional effect in MT than experiment 2, which uses differential attention within the receptive field. Our results are in agreement with a functional magnetic resonance imaging study showing attentional modulation located in a region believed to contain the human homologues of areas MT and MST<sup>21</sup> during a motion attention task. Modulations of responses to colours or oriented bars have also been described in the early stages of the ventral pathway in visual cortex<sup>22–24</sup>, although differences between attention inside and outside the receptive field, are not seen in all cases<sup>23</sup>.

The stronger attentional modulation that we found in MST compared with MT indicates that extraretinal influences may increase in successive levels of cortical processing, such that there is a progression from the purely sensory representations in the first stages of the retinocortical pathway to representations in later extrastriate cortex in which extraretinal factors have a

powerful, perhaps even dominating, influence. At the same time, our demonstration of robust attentional effects in MT—an area which receives direct input from primary visual cortical area V1 (refs 25, 26)—suggests that responses of neurons throughout much of the extrastriate cortex are substantially influenced by behavioural state, and that an understanding of visual information processing even in early extrastriate cortex requires approaches that do not concentrate solely on the sensory qualities of the visual input. □

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## Reversal of apoptosis by the leukaemia-associated E2A-HLF chimaeric transcription factor

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**THE E2A-HLF (for hepatic leukaemia factor) fusion gene, formed by action of the t(17;19) (q22;p13) chromosomal translocation, drives the leukaemic transformation of early B-cell precursors<sup>1–4</sup>, but the mechanism of this activity remains unknown. Here we report that human leukaemia cells carrying the translocation t(17;19) rapidly died by apoptosis when programmed to express a dominant-negative suppressor of the fusion protein E2A-HLF, indicating that the chimaeric oncoprotein probably affects cell survival rather than cell growth. Moreover, when introduced into murine pro-B lymphocytes, the oncogenic E2A-HLF fusion**

protein reversed both interleukin-3-dependent and p53-mediated apoptosis. The close homology of the basic region/leucine zipper (bZIP) DNA-binding and dimerization domain of HLF to that of the CES-2 cell-death specification protein of *Caenorhabditis elegans*<sup>5</sup> suggests a model of leukaemogenesis in which E2A-HLF blocks an early step within an evolutionarily conserved cell-death pathway<sup>6-9</sup>.

The effects of blocking E2A-HLF activity in t(17;19)-positive human leukaemia cells (UOC-B1 line) were analysed by a conditional dominant-negative strategy. The mutant protein selected, E2A-HLF(dn), lacks the AD1 *trans*-activation domain of E2A<sup>10,11</sup> and contains a defective HLF basic domain, with substitutions for six of the amino-acid residues critical for DNA binding (ARRRKNMAAK mutated to ASPRSYNMPPK; single-letter code). E2A-HLF(dn) formed heterodimers with E2A-HLF through its intact leucine-zipper domain, but these were unable to bind to DNA (data not shown). When leukaemic cells expressing E2A-HLF were transfected with a zinc-inducible E2A-HLF(dn) vector, expression of the mutant protein was highly dependent on the addition of zinc to the culture medium, whereas the intact protein was detected in cell lysates from all clones (immunoblot data not shown).

The effects of E2A-HLF(dn) expression were assessed in an electrophoretic mobility shift analysis (EMSA) using an oligonucleotide probe incorporating the HLF consensus-binding sequence. Of the four main DNA-protein complexes (A-D; Fig. 1) observed in nuclear extracts of UOC-B1 cells, the B

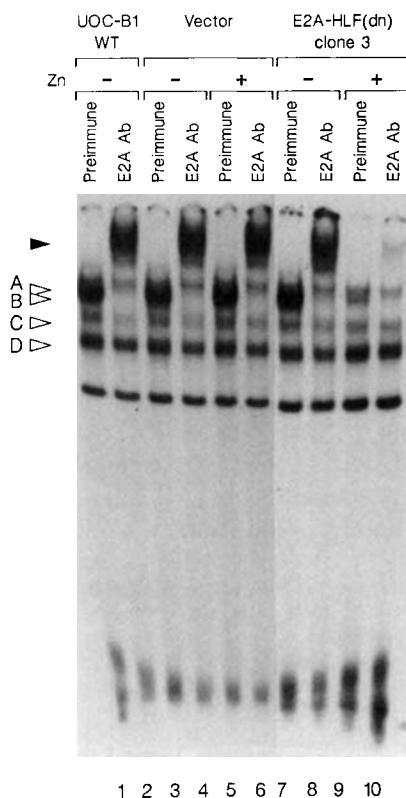


FIG. 1 DNA-binding properties of E2A-HLF proteins in t(17;19)-positive UOC-B1 cells, as demonstrated by EMSA assay with a <sup>32</sup>P-labelled oligonucleotide probe containing the HLF consensus binding sequence. The probe was incubated with nuclear extracts from control UOC-B1 cells (lanes 1 and 2), cells containing the empty pMT-CB6+ vector (lanes 3-6), and clone-3 cells with or without zinc induction of expression of a dominant-negative mutant of the E2A-HLF oncoprotein (E2A-HLF(dn), lanes 7-10). For antibody-perturbed gel-shift analysis, 0.2 µl of anti-E2A rabbit serum was added to the reaction mixtures in the even-numbered lanes.

complex was recognized by the E2A antibody, indicating that it contains the E2A-HLF protein (lanes 1 and 2). When expression of the E2A-HLF(dn) protein was induced with zinc in UOC-B1 clones, the levels of E2A-HLF protein-DNA complexes were reduced 10-fold, as shown for clone 3 (Fig. 1, lanes 7-10), indicating that overexpression of the mutant protein sequesters the oncogenic E2A-HLF protein into heterocomplexes that no longer bind the HLF DNA-binding consensus sequence. DNA binding by oncogenic E2A-HLF proteins was not affected when control UOC-B1 cells transfected with the empty pMT-CB6+ vector were cultured with zinc (Fig. 1, lanes 3-6).

The E2A-HLF(dn) protein profoundly reduced the growth rate and viability of UOC-B1 cells when it was overexpressed in each of the six independent clones tested (Fig. 2a). This effect persisted for 7 to 14 days, with rapid restoration of normal growth and survival properties after removal of zinc from the medium (Fig. 2a, right). Zinc does not seem to be toxic to UOC-B1 cells, as controls bearing the empty pMT-CB6+ vector grew normally in zinc-supplemented medium (Fig. 2a, left). To ensure that the antileukaemic effects of E2A-HLF(dn) were restricted to the inhibition of E2A-HLF, we programmed the t(17;19)-negative human Nalm-6 pre-B and Jurkat T leukaemia cell lines to express the mutant protein. Multiple clones isolated from each cell line expressed E2A-HLF(dn) in amounts that were equivalent to those seen in UOC-B1 cells; however, the cell growth rate and viability were unaffected (data not shown).

The basis for the altered growth and survival of UOC-B1 cells expressing E2A-HLF(dn) was investigated first by DNA flow cytometry. The results indicated similar cell-cycle phase distributions in cells whether or not they expressed the mutant protein; further experiments with bromodeoxyuridine (BrdU) labelling failed to reveal a difference in cell-cycle transit times (not shown). Thus the attenuated growth rates and survival of these cells could not be attributed to an effect on the cell-cycle machinery. Instead, an increased rate of cell death caused by the restoration of apoptotic signals in the absence of functional E2A-HLF was observed in zinc-treated cells expressing E2A-HLF(dn) (Fig. 2b). Induction of programmed cell death was confirmed by electron microscopy, which demonstrated unequivocal apoptotic changes, including nuclear fragmentation, chromatin condensation, and perinuclear-membrane blebbing, with preservation of cytoplasmic organelles and membranes (Fig. 2c).

These observations indicate that E2A-HLF probably contributes to leukaemogenesis by subverting genetic pathways responsible for the apoptotic death of pro-B cells. To test this hypothesis directly, we conditionally expressed intact E2A-HLF proteins in Baf-3 cells, an interleukin (IL)-3-dependent line of murine pro-B lymphocytes<sup>12</sup>. Three clones were isolated, each expressing high levels of zinc-inducible E2A-HLF proteins in the presence of 100 µM ZnSO<sub>4</sub>; a fourth isolate (clone 32) expressed the intact protein at fourfold lower levels (immunoblot data not shown).

The chimaeric E2A-HLF protein had no effect on the growth rates of these four clones as long as the cells were cultured in the presence of IL-3 (Fig. 3). When the growth factor was removed, cells cultured in the absence of zinc died apoptotically within 2 to 3 days, whereas those expressing E2A-HLF survived for extended periods. Most cells derived from clones with the highest levels of E2A-HLF expression survived for more than 2 weeks and were able to resume normal exponential growth when IL-3 was reintroduced into the medium (Fig. 3a). The wild-type HLF protein had no effect on Baf-3 cell survival in the absence of IL-3 when expressed at levels comparable to those of E2A-HLF with the same zinc-inducible vector (data not shown). Thus, in addition to the bZIP DNA-binding domain contributed by the HLF protein, the E2A activator domain was essential for the anti-apoptotic effects of the chimaeric oncoprotein.

Wild-type Baf-3 cells continue to progress through the cell cycle for a short time after IL-3 withdrawal, but within 24 hours they begin to accumulate in G1 phase and to exhibit DNA fragmentation<sup>13</sup>. The E2A-HLF<sup>+</sup> Baf-3 cells in this study showed exactly the



**b**

UOC-B1 cells + zinc (vector)

UOC-B1 cells + zinc (E2A-HLF (dn))

DNA content

200

100

0

Intact Apoptotic

Log fluorescence intensity

10<sup>0</sup> 10<sup>1</sup> 10<sup>2</sup> 10<sup>3</sup> 10<sup>4</sup>

10<sup>0</sup> 10<sup>1</sup> 10<sup>2</sup> 10<sup>3</sup> 10<sup>4</sup>

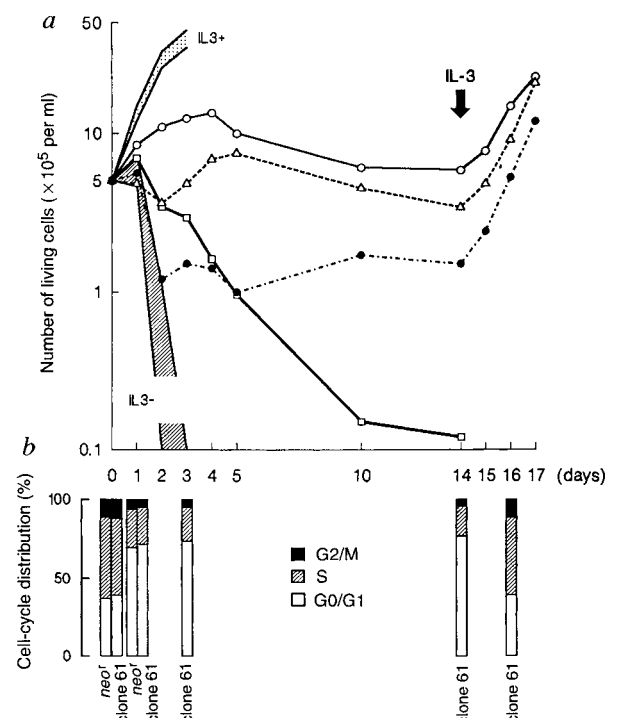
**c**

Zn -

Zn +

Figure 2 consists of two panels, b and c. Panel b contains two flow cytometry histograms. The y-axis is labeled 'DNA content' with values 0, 100, and 200. The x-axis is labeled 'Log fluorescence intensity' with values 10<sup>0</sup>, 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, and 10<sup>4</sup>. The left histogram is titled 'UOC-B1 cells + zinc (vector)' and the right is 'UOC-B1 cells + zinc (E2A-HLF (dn))'. Both histograms have a vertical line at 10<sup>1</sup> separating the 'Intact' region (left) from the 'Apoptotic' region (right). The histograms show a dense population of cells in the 'Intact' region and a smaller population in the 'Apoptotic' region. Panel c contains two electron micrographs. The left micrograph is labeled 'Zn -' and shows several cells with normal morphology, including large nuclei and clear cytoplasm. The right micrograph is labeled 'Zn +' and shows cells with apoptotic features, such as condensed nuclei and fragmented chromatin.

FIG. 3 *a*, Cell-growth curve and *b*, cell-cycle phase distribution of Baf-3 cells induced to express the E2A-HLF protein, together with control (neomycin-resistant) cells. Cells growing exponentially in media for 48 h with (Zn+) or without (Zn-) zinc were adjusted to  $5 \times 10^5$  cells per ml on day 0, and cultured with or without IL-3. The growth factor was reintroduced to the IL3- cultures on day 14 (arrow). Shaded regions indicate the ranges of cell counts for the IL3+ (all cell types, Zn+/-) and IL3- (*neo*' Zn-/+; clones 22, 32, 52 and 61 Zn-) growth conditions. Open circles, clone 61; open triangles, clone 22; filled circles, clone 52; open squares, clone 32 (all IL3-, Zn+). *b*, Bar graphs showing cell-cycle phase distributions, calculated from DNA histograms obtained by propidium iodide staining and flow cytometry. Also shown are cell-cycle phase distributions for the empty vector, neomycin-resistant controls (*neo*'; days 0 and 1) and clone 61 cells expressing E2A-HLF (days 0, 1, 3, 14 and 16), all cultured in zinc-containing media.



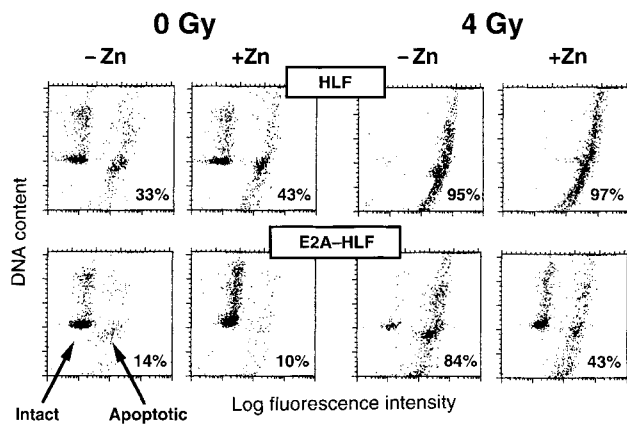


FIG. 4 Effects of zinc-inducible HLF or E2A-HLF overexpression on radiation-induced apoptosis of Baf-3 cells cultured in the absence of IL-3. Culture conditions were as in Fig. 3, except that zinc pretreatment was for only 24 h. Pretreated cells were washed and resuspended in IL-3-free medium containing zinc and immediately exposed to radiation (0 or 4 Gy). Cells mock-treated with zinc are also shown for each condition. Cells were end-labelled with biotinylated dUTP (deoxyuridine triphosphate) using terminal deoxynucleotidyl transferase 24 h postirradiation, and stained with fluorescein isothiocyanate (FITC) conjugated avidin (x-axis)<sup>13</sup>. Cellular DNA was then counterstained with propidium iodide (y-axis). The value in the lower right-hand corner of each panel is the percentage of apoptotic (TdT-positive) cells in each population. HLF-positive cells (clone 2) were generated after stable transfection of Baf-3 cells with the pMT-CB6+/HLF vector and selection in G418-containing medium. The cells produced HLF protein in response to zinc treatment by immunoblot analysis (data not shown).

same pattern of G1-phase accumulation as did controls after 24 hours. However, they maintained that pattern throughout the two-week period of culture without IL-3, whereas control cells rapidly died by apoptosis (compare clone 61 expressing E2A-HLF and control cells in Fig. 3b). Consistent with their resumption of exponential growth, E2A-HLF-expressing cells that survived growth-factor withdrawal regained normal cell-cycle distributions after IL-3 was returned to the medium. Thus E2A-HLF protects Baf-3 cells from the apoptotic effects of IL-3 withdrawal, but does not replace the cell-cycle stimulatory effects of the growth factor. Programmed expression of the BCL2 protein in Baf-3 cells delayed apoptotic cell death by 24–48 hours in the absence of IL-3, as in an earlier study<sup>14</sup>, but did not increase survival by more than 10 days, indicating a clear distinction between these cells and those overexpressing E2A-HLF (data not shown).

E2A-HLF overexpression also protected Baf-3 cells from p53-mediated apoptotic death induced by ionizing radiation in cultures lacking IL-3 (Fig. 4; lower right panel)<sup>13,14</sup>. In contrast, conditional expression of HLF failed to provide any protection from apoptosis induced by either growth-factor withdrawal or radiation (upper panels), demonstrating that the protective effects of the chimaeric protein are not merely due to inappropriate expression of HLF activity in Baf-3 cells. Thus expression of E2A-HLF in t(17;19)-positive leukaemic cells probably protects against both radiation- and drug-induced apoptosis, accounting for the refractoriness of these cases to treatments generally effective against pro-B-cell acute leukaemias<sup>15</sup>.

Genetically controlled suicide programs are important in cell fate decisions that govern homeostasis in multicellular organ-

isms<sup>16–18</sup>. Hence genetic changes that inappropriately extend cell survival could be expected to contribute to neoplastic transformation. This concept is supported by studies of the *BCL2* oncogene in follicular lymphoma, the product of which inhibits apoptosis, leading to an increase in cell number independent of any effect on cell proliferation<sup>19–22</sup>. Although the effector mechanisms of apoptosis in developing mammalian cells are still unclear<sup>23</sup>, recent observations indicate their similarity to those operating in the nematode *C. elegans*<sup>6,24</sup>. Briefly, all programmed cell deaths that occur during *C. elegans* development require the combined activities of CED-3 (a cysteine protease) and CED-4 (a protein with unknown biochemical function). Cell survival in this organism also depends on CED-9, an antagonist of CED-3 and CED-4. Mammalian homologues of these proteins include BCL2 and BCLx (CED-9)<sup>25</sup>, as well as interleukin-1 $\beta$ -converting enzyme (ICE) and its family members (CED-3)<sup>26</sup>. CES-2, a recently isolated *C. elegans* transcription factor in the bZIP superfamily, has high amino-acid identity with HLF<sup>5</sup>, and regulates the programmed suicide of two superfluous cells produced by the last division of specific neuronal progenitors in developing nematodes<sup>7</sup>. We suggest that E2A-HLF dominantly interferes with a CES/CED-like apoptotic pathway that censors immature B lymphocytes which have failed to productively rearrange their antigen receptor molecules, have sustained DNA damage, or have not received the appropriate survival or differentiation signals<sup>24</sup>. As HLF is not normally expressed in lymphoid cells, the chimaeric oncoprotein probably competes with other transcription factors that bind to the HLF consensus sequence and normally have a CES-2-like function in the regulation of pro-B-cell mortality. □

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# Transcriptional regulator of programmed cell death encoded by *Caenorhabditis elegans* gene *ces-2*

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**THE *ces* (for cell-death specification) genes of the nematode *Caenorhabditis elegans* control the cell-death fate of individual cell types and are candidates for being the regulators of an evolutionarily conserved general pathway of programmed cell death<sup>1-4</sup>. Here we present what we believe is the first molecular characterization of a *ces* gene. We cloned the gene *ces-2*, which is required to activate programmed cell death in the sister cells of the serotonergic neurosecretory motor (NSM) neurons, and found that *ces-2* encodes a basic region leucine-zipper (bZIP) transcription factor. The CES-2 protein is most similar to members of the PAR (proline- and acid-rich) subfamily of bZIP proteins and has DNA-binding specificity like that of PAR-family**

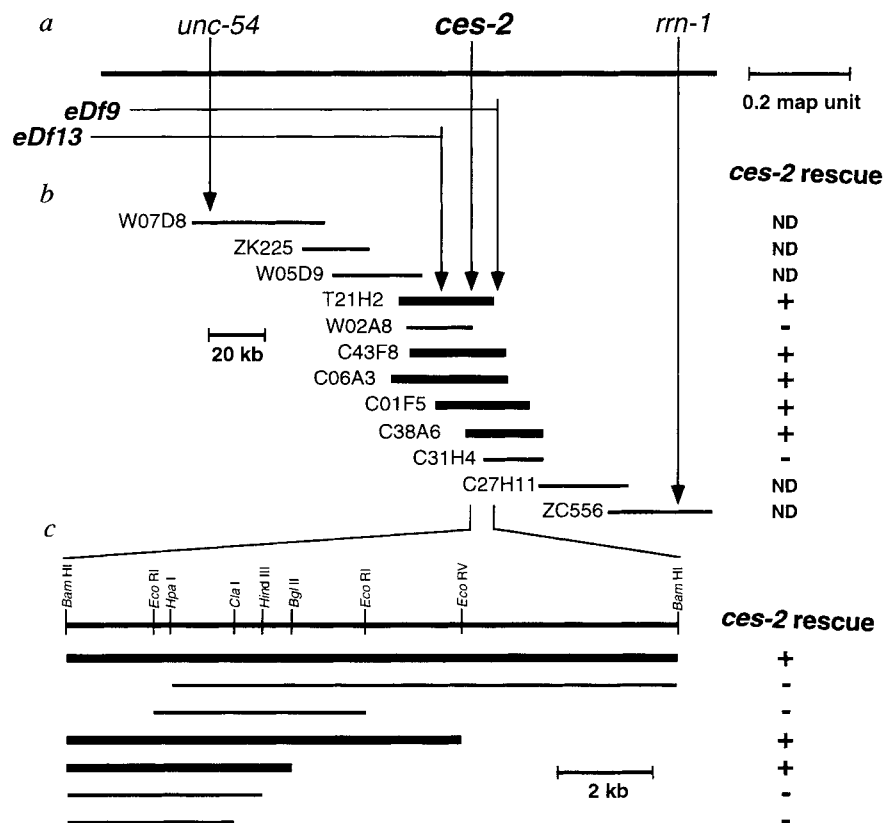
proteins. An oncogenic form of the mammalian PAR-family protein, hepatic leukaemia factor (HLF), is reported to effect programmed cell death in mammalian cells<sup>5</sup>. On the basis of these observations, we suggest that some CES-2/PAR family transcription factors are evolutionarily conserved regulators of programmed cell death.

We used a positional approach to clone *ces-2*. First, by physically mapping deficiency endpoints, we localized *ces-2* to a roughly 25-kilobase (kb) region (Fig. 1a, b). Germline transformation experiments showed that cosmids spanning this interval were able to rescue the *ces-2* mutant phenotype (Fig. 1b). We narrowed the rescuing activity to a 4.4-kb fragment (Fig. 1c) and determined the sequence of this fragment. Analysis of this sequence using the program Genefinder (L. Hillier, personal communication) predicted a single gene. We isolated a set of complementary DNAs encoded in the rescuing interval (Fig. 2a). These cDNAs confirmed the structure of the gene as predicted by Genefinder. Rapid amplification of cDNA ends (RACE) and cDNA analyses identified an SL1 *trans*-splice leader, which is found at the 5' end of many *C. elegans* transcripts<sup>6</sup>, confirming that we had defined a complete *ces-2* transcription unit. Genomic subclones lacking portions of this transcription unit failed to rescue *ces-2* (Figs 1c, 2a). Northern analysis revealed a single product of 1.1 kb, consistent with the sizes of the cDNAs (data not shown). We found that *ces-2* messenger RNA was detectable only during embryogenesis, consistent with the role of *ces-2* in controlling the embryonic deaths of the NSM sisters (data not shown)<sup>1,7</sup>.

DNA sequence analysis indicates that *ces-2* encodes a single protein of 211 amino acids (Fig. 2b). We found that regions of the predicted CES-2 protein were similar to the basic and leucine-zipper domains of proteins of the bZIP family of transcription

**FIG. 1** Cloning of *ces-2*. **a**, Genetic map of the right end of linkage group I (LGI). The *ces-2* gene is located between *unc-54* and the end of LGI as defined by the ribosomal RNA cluster, *rrn-1*. *eDf13* complements *ces-2*, whereas *eDf9* does not complement *ces-2* (ref. 1). **b**, Physical map of the right end of LGI (ref. 25). Positions of *unc-54*, *rrn-1* and the right breakpoints of *eDf9* and *eDf13* indicated by arrows. Cosmids in bold were tested and rescued *ces-2*. Rescue results: +, rescue; -, no rescue; ND, not determined. **c**, Subclone rescue of *ces-2*. Subclones in bold rescued *ces-2*.

**METHODS.** Standard molecular biology procedures were followed<sup>26</sup>. Genomic Southern blots of DNA from strains balanced for the deficiency of interest were probed with cosmids from the *C. elegans* physical map<sup>25</sup> spanning the interval from *unc-54* to the ribosomal DNA cluster to detect regions of DNA deleted by the deficiencies (data not shown). Candidate cosmids were then used for germline transformation<sup>27</sup>. To assay for *ces-2* rescue we injected test DNA into *ces-2*(*n732*); *unc-76*(*e911*) animals using the *unc-76* rescuing plasmid pU76-16B (L. Bloom and H.R.H., unpublished results) as a transformation marker. Stably transmitting lines were established, grown at 25.5 °C, and tested for absence of NSM-sister survival by direct observation using Nomarski optics<sup>1</sup>. At least 50 animals were scored for each line. Because the *n732* allele is temperature sensitive<sup>1</sup>, we controlled for temperature fluctuations by growing control strains in parallel and scoring them with the newly generated transgenic strains. Lines that resulted in



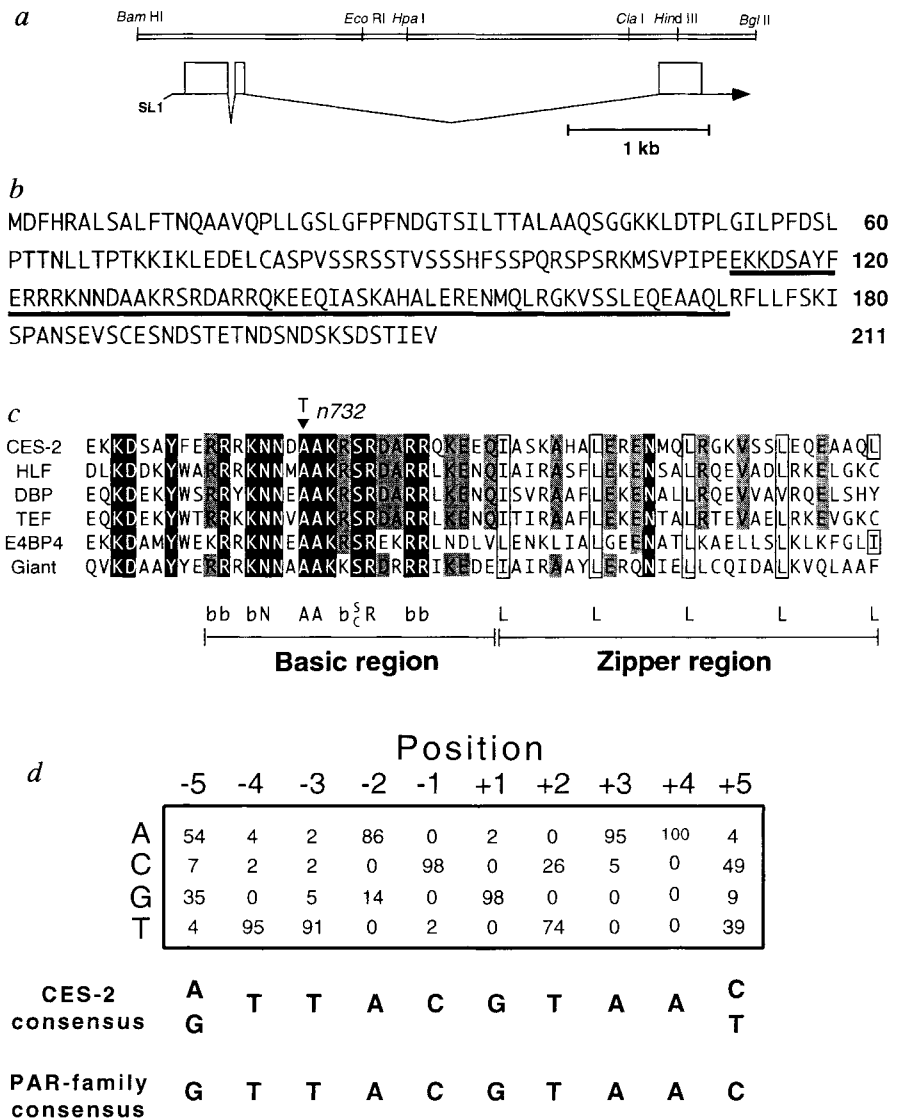
NSM sister survival frequencies of less than 25% of controls were considered rescued. Cosmids were injected at 8 ng  $\mu\text{l}^{-1}$  each. Plasmids and pU76-16B were injected at 50 ng  $\mu\text{l}^{-1}$ .

FIG. 2 Sequence analysis of *ces-2*. *a*, The *ces-2* intron-exon structure. Open boxes, coding regions; lines, untranslated regions; arrow, direction of transcription; SL1, SL1 *trans*-spliced leader. *b*, Deduced CES-2 protein sequence (single-letter code). The bZIP domain is shown underlined. *c*, Alignment among the basic and zipper regions of CES-2, the PAR-family proteins HLF, DBP, and TEF, the human protein E4BP4, and the *Drosophila* protein Giant. Identities among all proteins aligned are in black; residues that are identical among CES-2 and the PAR family proteins are in grey. The triangle above the CES-2 sequence indicates the residue altered (Ala to Thr) in the *n732* mutant. Hydrophobic residues of the zipper heptad repeats are boxed. Below the alignment is the bZIP consensus (b is the basic residue)<sup>8</sup>. *d*, Base frequencies (%) of 54 oligonucleotides selected by binding to CES-2 protein and derived consensus binding site. Shown for comparison is the PAR-family protein consensus binding site<sup>17-19</sup>.

**METHODS.** We determined the sequence of *ces-2* genomic DNA and cDNAs by a shotgun procedure<sup>26</sup> using an ABI 373A sequencer. We isolated cDNAs encoded in the rescuing fragment from a mixed-stage  $\lambda$ ZAP cDNA library and an embryonic  $\lambda$ gt11 library<sup>28,29</sup>. For RACE we used the 5' RACE system (GIBCO-BRL). To identify base changes we determined the DNA sequences of polymerase chain reaction (PCR) amplification products of DNA isolated from wild-type and *ces-2(n732)* animals. The resulting products were purified using agarose gels and then used directly in sequencing reactions. All sequence was determined from both strands and, apart from the *n732* mutation, was identical to the sequence determined from cDNAs and genomic clones. For binding-site selection we used the method described in ref. 17. The GenBank accession number for *ces-2* sequences is U60979.

factors<sup>8</sup> (Fig. 2c). The basic and zipper domains of CES-2 are most similar to those found in a bZIP subfamily, the PAR proteins. This subfamily, defined by the mammalian genes for HLF<sup>9,10</sup>, albumin D-box binding protein (DBP)<sup>11</sup>, thyrotroph embryonic factor (TEF)<sup>12</sup>, and the avian gene for vitellogenin gene-binding protein (VBP)<sup>13</sup>, is named for the conserved proline- and acid-rich domain, which is of unknown function. Whereas the CES-2 protein has a region relatively rich in proline residues (Fig. 2b), CES-2 does not possess the conserved sequence of proline and acidic residues seen in the vertebrate PAR domains. The human gene E4BP4/NF-IL3A (refs 14, 15) and the *Drosophila* gene *giant*<sup>16</sup> have a bZIP domain almost as similar to that of CES-2 as the PAR proteins noted above (Fig. 2c). Outside the bZIP domain, CES-2 is not similar to any proteins in current databases.

To see whether CES-2 is similar to these bZIP proteins in its target specificity, we determined the consensus binding site of CES-2. By using a bacterially-produced glutathione *S*-transferase-CES-2-bZIP domain fusion protein to select binding sites from a pool of random oligonucleotides<sup>17</sup>, we found that CES-2 has the same binding-site consensus, RTTACGTAAAY (R = G or A; Y = C or T) (Fig. 2d), as do the PAR-family proteins<sup>17-19</sup>. This site is similar to, but distinct from, the Giant<sup>19</sup> and E4BP4 (ref. 14) consensus sites. Thus, on the basis of both sequence similarity and



binding-site specificity, CES-2 is most closely related to the PAR family of bZIP proteins.

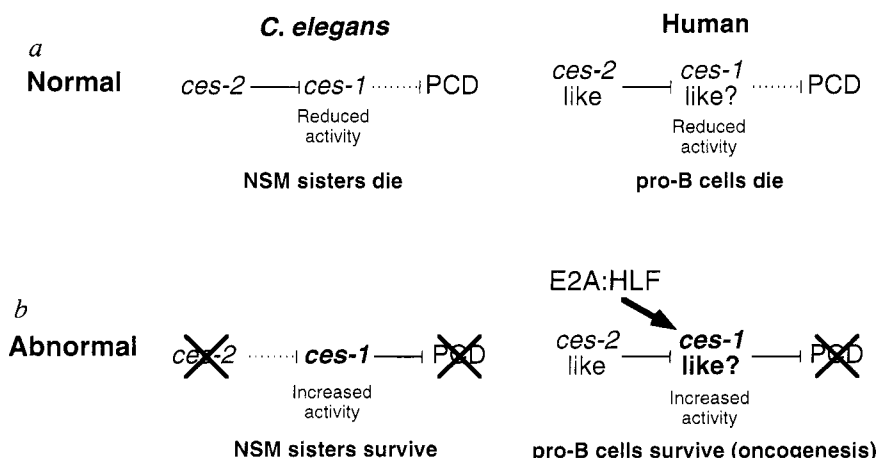
There exists a single mutant allele of *ces-2*, *n732*, which results in a partial loss of *ces-2* function<sup>1</sup>. We found a single-base transition mutation in the *ces-2* coding sequence in *n732* mutant animals (Fig. 2c). This mutation should change alanine 129 (GCA) to a threonine (ACA). This alanine is one of the most highly conserved residues in the bZIP basic domain<sup>8</sup>, and it seems from structural and genetic analyses of the corresponding alanines in the yeast bZIP protein GCN4 and the mammalian bZIP heterodimer Fos-Jun that this change is likely to lead to a severe reduction in CES-2 function<sup>20-22</sup>.

Genetic studies of *ces-2* indicate that this gene activates programmed cell death indirectly, by negatively regulating the activity of a second gene, *ces-1*, which in turn negatively regulates the activities of cell-killing genes<sup>1</sup>. Because *ces-2* encodes a transcription factor, such negative regulation might be mediated by direct transcriptional repression of *ces-1*. The CES-2-related proteins E4BP4 and Giant have been shown to act as transcriptional repressors<sup>14,16,23</sup>.

We have shown that CES-2 and the PAR-family proteins have similarities in amino-acid sequence and possess the same target specificity, leading us to question whether these proteins are



FIG. 3 Model of the cell-death activities of the CES-2 protein and the E2A:HLF oncoprotein. **a**, In normal *C. elegans* NSM sister cells, or in human pro-B cells, the activity of *ces-2* or a human *ces-2* homologue represses (barred line) the activity of a cell-death protecting gene, *ces-1* in *C. elegans* or a human *ces-1*-like gene. This repression prevents the *ces-1*-like gene from inhibiting programmed cell death (PCD) (broken line), and the cell dies. **b**, In *ces-2* loss-of-function mutants the reduction of *ces-2* activity leads to an increase in *ces-1* activity (bold), which in turn blocks programmed cell death. In human pro-B cells harbouring the E2A:HLF fusion protein, this protein activates (arrow) the *ces-1*-like activity, leading to a block in programmed cell death<sup>5</sup> and contributing to oncogenesis.



involved in an evolutionarily conserved process. As genetic evidence suggests that CES-2 functions as a negative regulator, possibly as a transcriptional repressor, of a gene required for cell survival, we predict that a transcriptional activator with the same binding specificity as CES-2 would activate such a cell-survival gene and thus inhibit programmed cell death. A naturally occurring protein with these properties has been characterized. It has been demonstrated<sup>5</sup> that an oncogenic protein that is created by fusion of the transactivation domain of the transcription factor E2A with the bZIP domain of the CES-2/PAR-family member HLF<sup>9,10,24</sup> can inhibit programmed cell death in mammalian cells. This fusion, which is generated by a t(17;19) translocation found in a form of the pro-B-cell cancer, acute lymphoblastic leukaemia (ALL)<sup>9,10</sup>, retains the HLF-binding specificity and is a potent transactivator in lymphocytes<sup>17,18</sup>. This finding is in accordance with our molecular and genetic interpretation of *ces-2*. By analogy, we predict that in pro-B cells there is a CES-2-like protein that functions to regulate negatively a cell-survival gene and allow programmed cell death to occur in these cells (Fig. 3). The E2A–HLF fusion protein presumably upregulates this survival gene, blocking programmed cell death (Fig. 3). We further predict that this cell-survival factor is encoded by a conserved downstream target of the CES-2/PAR-family proteins—a CES-1-like protein—the misexpression of which inhibits programmed cell death and results in ectopic cells in *C. elegans ces-2* mutants and oncogenesis in mammals. □

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## smoothened encodes a receptor-like serpentine protein required for hedgehog signalling

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MEMBERS of the Hedgehog family of secreted proteins control a number of important inductive interactions in the development of both vertebrates and *Drosophila*<sup>1</sup>, but little is known about the ways in which their signalling activities are transduced. In *Drosophila*, *hedgehog* is one of the segment-polarity genes, mutations of which disrupt the pattern and polarity of individual embryonic segments<sup>2</sup> and their adult derivatives<sup>3</sup>; several of these genes have been implicated in transduction of the *hedgehog* signal<sup>4–6</sup>. Here we show that the segment-polarity gene *smoothened* is required for the response of cells to *hedgehog* signalling during the development of both the embryonic segments and imaginal discs. Sequence analysis of the *smoothened* transcription unit reveals a single open reading frame encoding a protein with seven putative transmembrane domains. This structure is typical of G-protein-coupled receptors, suggesting that the *Smoothened* protein may act as a receptor for the Hedgehog ligand.

In the *Drosophila* embryo, spatially restricted expression of the *wingless* (*wg*) signalling protein, which is essential for the normal patterning of each segment (reviewed in ref. 7), is controlled by the localized activity of *hedgehog* (*hh*) in neighbouring cells (reviewed in ref. 1). Expression of *hh* is in turn maintained by *wg* activity, and these mutual regulatory interactions stabilize the expression domains of the two genes and hence the parasegment boundaries defined by their interfaces (reviewed in ref. 8). In the

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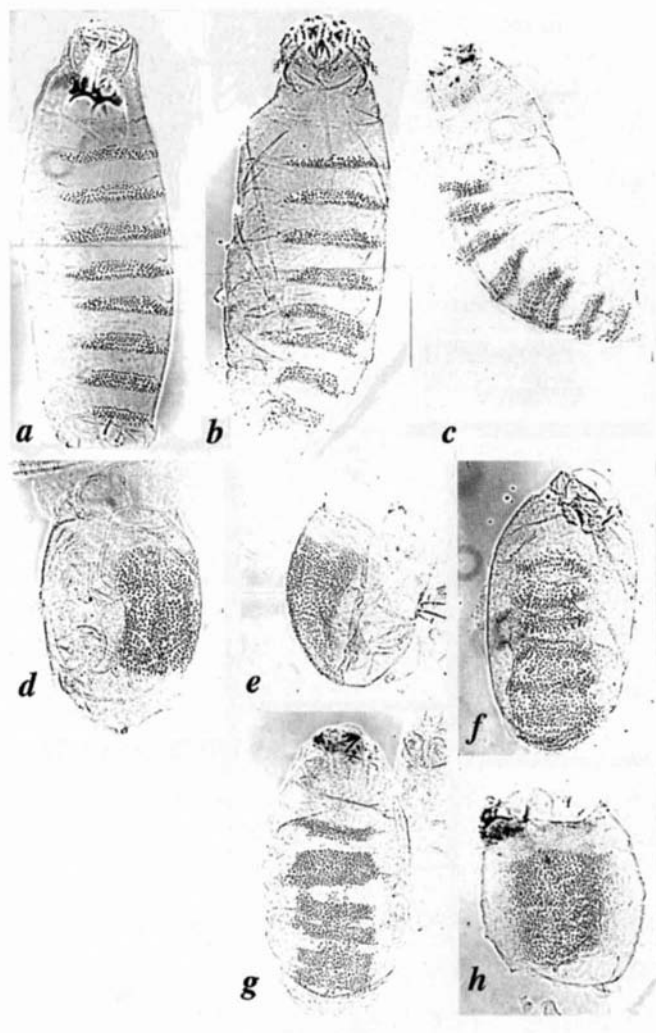
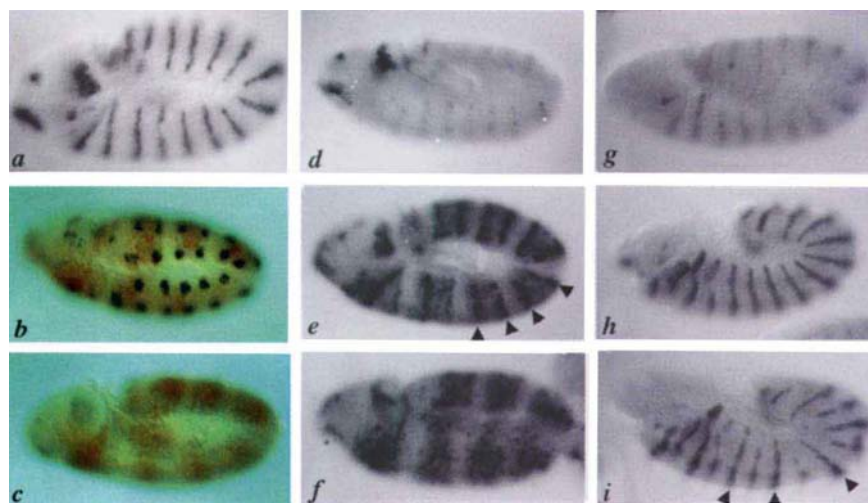


FIG. 1 Phase-contrast images of the ventral cuticle of pharate first instar larvae; anterior is to the top. *a*, Wild type. *b*, *smo*<sup>IX43</sup>, raised at 25 °C. Naked cuticle is deleted sporadically between adjacent denticle belts. *c*, *smo*<sup>IX43</sup>, raised at 18 °C. Naked cuticle is replaced by denticle belts with reversed polarity. *d*, *smo*<sup>-</sup> embryo derived from *smo*<sup>D16</sup> germline clone females. All naked cuticle and polarity is lost; this phenotype is typical of all *smo* alleles (three) analysed in germline clone analysis. *e*, *hh*<sup>13C</sup> (severe phenotype<sup>26</sup>); the phenotype is virtually identical to that of a *smo*<sup>-</sup> embryo in *d*. *f*, *wg*<sup>CX4</sup> (null allele<sup>12</sup>); note the complete loss of naked cuticle but vestiges of polarity, in contrast to in *smo*<sup>-</sup> and *hh* null mutants remain visible. *g*, *smo*<sup>-</sup>;hGAL4-UASwg; naked cuticle is partially restored in alternate segments. *h*, *smo*<sup>-</sup>;hGAL4-UAShh; phenotype is indistinguishable from that of *smo*<sup>-</sup>.

**METHODS.** All *smo* alleles have been described<sup>9</sup> except *smo*<sup>D16</sup>. This allele and two others (*smo*<sup>F5</sup> and *smo*<sup>F11</sup>) were isolated in an F<sub>2</sub> lethal screen following γ-ray mutagenesis. Isogenized *cn bw sp* males were irradiated with 4,000 Rad emitted by a <sup>60</sup>Co source and mated to wild-type females. Individual F<sub>1</sub> males (12,000) were test-crossed to *smo*<sup>IX43</sup> *cn bw sp*/CyO females and the F<sub>2</sub> progeny screened for the absence of white-eyed flies. Germline clone females were generated by the dominant female sterile technique either by irradiating larvae with γ-rays or using flippase-induced mitotic recombination<sup>27</sup>. Ectopic expression of *wg* and *hh* was induced by the GAL4-UAS system using the *hairy* GAL4 enhancer trap, which expresses GAL4 in every other segment<sup>28</sup>. *hGAL4* UASwg<sup>6</sup>, UAShh and *hGAL4* chromosomes have been described.

FIG. 2 Expression of *wg*, *hh* and *En* in wild-type and mutant embryos. *a*, Expression of *wg* in wild-type stage 10. *b*, Expression of *wg* (blue/black) and *hh* (red) in *hGAL4*-UAShh embryo. The ectopic expression of *hh* induces the broadening of *wg* in all segments because of the overlap of endogenous *wg* and ectopic *hh*<sup>6</sup>. *c*, *wg* and *hh* in *smo*<sup>-</sup>;hGAL4 UAShh; expression of *wg* in the segmented germband is completely lost as in *smo*<sup>-</sup> embryos (see *d*). *d*, *wg* in *smo*<sup>-</sup> stage 10; all *wg* expression disappears from the segmented germband. *e*, *hGAL4* UASwg stage 10 embryo; ectopic and endogenous (arrowheads) *wg* stripes are seen to overlap, with the *h* driven *wg* expression spanning every other segment. *f*, *smo*<sup>-</sup>;hGAL4 UASwg stage 10 embryo; ectopic but no endogenous *wg* expression is detected. *g*, Stage 10 *smo*<sup>-</sup>; *En* is lost from the ectoderm in all thoracic and abdominal segments. *h*, Stage 11 *hGAL4* UASwg embryo, showing slightly broadened stripes of *En* (compared to wild-type embryos). *i*, *smo*<sup>-</sup>;hGAL4 UASwg stage 10. *En* expression is maintained in alternate segments (arrowheads), corresponding to the segments where *wg* expression is driven by *hGAL4*. In the intervening stripes, some *En* is rescued, presumably due to the paracrine action of the misexpressed *wg*.

**METHODS.** Embryos were collected for one hour and aged for the appropriate times. Fixations, *in situ* hybridizations and antibody stainings (for *En*,

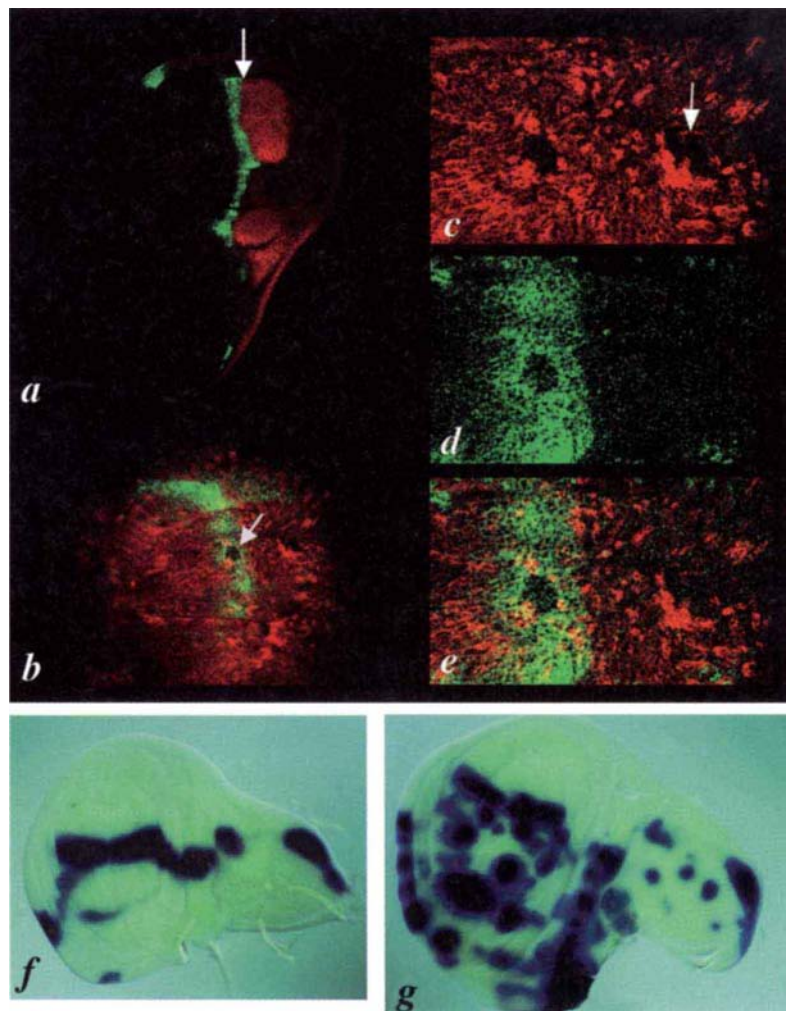


using monoclonal 4D9) have been described<sup>4,12</sup>. Double-labelling with an *hh* fluorescein-labelled and a *wg* digoxigenin probe was performed with two sequential alkaline phosphatase substrates, BCIP/NBT and Vector red; the enzyme was inactivated between the steps by treatment with 0.2 M glycine, pH 2.5. Genotypes are described in Fig. 1 legend.



FIG. 3 Stainings of third instar imaginal discs. **a**, Dpp expression (green) anterior to the compartment boundary is shown, as marked by the expression of En (red) throughout the posterior compartment (as *hh*). **b**, Wing-blade area stained for Dpp (green) and Myc epitope (red). Arrow indicates a *smo*<sup>-</sup> clone in the *dpp* expression region. **c–e**, Details of disc in **b**. Similar clones within the *dpp* domain were found in six cases among 350 dissected progeny, of which 1/8 were of the correct genotype. Loss of Myc staining represents cells that have lost *smo*<sup>+</sup> activity, whereas nearby cells expressing high levels of Myc represent the sister clone carrying two copies of the *myc* construct. In **c**, arrow points to a *smo*<sup>-</sup> clone within the posterior compartment. The absence of green staining (Dpp) corresponds cell for cell (**e**) with the absence of red staining (*smo*<sup>-</sup>). **f**, *dpp* expression; **g**, as in **f**, except that clones lacking *smo*<sup>+</sup> and the catalytic subunit of PKA were induced; these express *dpp* ectopically in the anterior compartment. Note the expansion of the anterior compartment owing to the overexpression of *dpp*<sup>29</sup>.

**METHODS.** Imaginal discs were dissected in PBS on ice and fixed in PBS/4% paraformaldehyde. For **a**, discs were collected from *dpp*LacZ animals and for **c–e**, from a cross of *smo*<sup>D16</sup> *ck* FRT/CyO females with Hs-FLP; FRT NM31E/CyO males, the progeny of which were heat-shocked at second-instar stage<sup>16</sup>. For **f** and **g**, discs were dissected from a cross of *smo*<sup>D16</sup> DCO<sup>H2</sup> FRT/CyO females with Hs-FLP; FRT *dpp*LacZ/CyO males, the progeny heat-shocked at several stages during larval development. Discs were stained with anti-En, anti-Dpp<sup>30</sup>, anti  $\beta$ -galactosidase (Promega) and anti-Myc epitope antibodies or stained for  $\beta$ -galactosidase activity using X-gal, following standard procedures.



absence of either gene activity, these boundaries disappear and all patterning and polarity of the segments is lost (Fig. 1e,f). Embryos homozygous for mutations of the *smoothed* (*smo*) locus (formerly named *smooth*)<sup>9</sup> exhibit phenotypes similar to those of weak alleles of *hh* or *wg*, affecting the patterning of each segment in a highly variable manner (Fig. 1a–c). In all cases, this phenotype is stronger at 18 °C than at 25 °C, suggesting that the variability may be due to the hypomorphism of each mutant *smo* allele. However, such variability might also be accounted for if maternally derived *smo* partially compensates for the loss of zygotic gene activity. To investigate this possibility, we generated mosaic females lacking wild-type *smo* alleles in their germ line (*smo*<sub>gic</sub>: *smo* germline clones) and analysed the phenotype of homozygous *smo* embryos from such *smo*<sub>gic</sub> females (hereafter designated *smo*<sup>-</sup> embryos). These *smo*<sup>-</sup> embryos display an invariant phenotype almost indistinguishable from that of *hh* null alleles (Fig. 1d–f): thus significant amounts of *smo* product are contributed to the egg during oogenesis, although this contribution is neither sufficient (as *smo* homozygous from heterozygous females die) nor necessary (as *smo* heterozygotes derived from *smo*<sub>gic</sub> females survive) for normal development.

The strong similarity between the *hh* null and *smo*<sup>-</sup> phenotypes is suggestive of a role for *smo* in *hh* signalling, and consistent with this, *smo*<sup>-</sup> embryos lack *wg* transcription at their parasegment boundaries (Fig. 2a,d). As *wg* transcription also disappears in *wg* mutant embryos<sup>10–12</sup>, however, this finding does not in itself exclude a role for *smo* in *wg* signalling. To address this issue directly, we examined the ability of each signal to function in the absence of *smo* activity by expressing either gene ectopically under the control of heterologous regulatory elements in *smo*<sup>-</sup> embryos. Expression of *wg* in alternate parasegments of a *smo*<sup>-</sup> embryo

results in the partial restoration of posterior naked cuticle in alternate segments (Fig. 1g); by contrast, expression of *hh* under identical conditions has no effect on the cuticular phenotype of a *smo*<sup>-</sup> embryo (Fig. 1h). These findings suggest that *smo* is required for the activity of *hh* but not of *wg*. To confirm this inference, the expression of *engrailed* (*en*) and *wg*, targets of *wg* and *hh* activity respectively, were analysed in each type of embryo. Whereas in a wild-type background, misexpression of *hh* induces ectopic *wg* expression<sup>5</sup>, in *smo*<sup>-</sup> embryos such *hh* misexpression has no effect on *wg* transcription (Fig. 2b,c), as would be expected if *smo* is required for *hh* signalling. In contrast, ectopic *wg* expression in *smo*<sup>-</sup> embryos restores the expression of *en* (Fig. 2g–i), confirming that *wg* signalling can occur in the absence of *smo* activity. Interestingly, however, transcription of the endogenous *wg* gene is not itself restored (Fig. 2e,f), implying that, contrary to previous assertions<sup>10,13</sup>, *wg* activity alone is not sufficient to maintain its own expression.

In the developing imaginal discs, expression of *hh* is restricted to cells of the posterior lineage compartment and, as in the embryo, its activity is required by neighbouring anterior compartment cells for the transcription of other signal-encoding genes (reviewed in ref. 14). In the case of the wing disc, the principal target of *hh* activity is the *decapentaplegic* (*dpp*) gene, expression of which is restricted to a thin stripe of cells running along the anterior side of the compartment boundary<sup>15</sup> (Fig. 3a). To determine whether *smo* is required for this *hh*-dependent expression of *dpp*, we used flippase-induced somatic recombination<sup>16</sup> to generate mosaic imaginal discs in which small clones of cells lack wild-type *smo* activity. When such clones are located anterior to the stripe of *dpp*-expressing cells or anywhere in the posterior compartment (Fig. 3c), the expression of *dpp* along the border is unaffected.

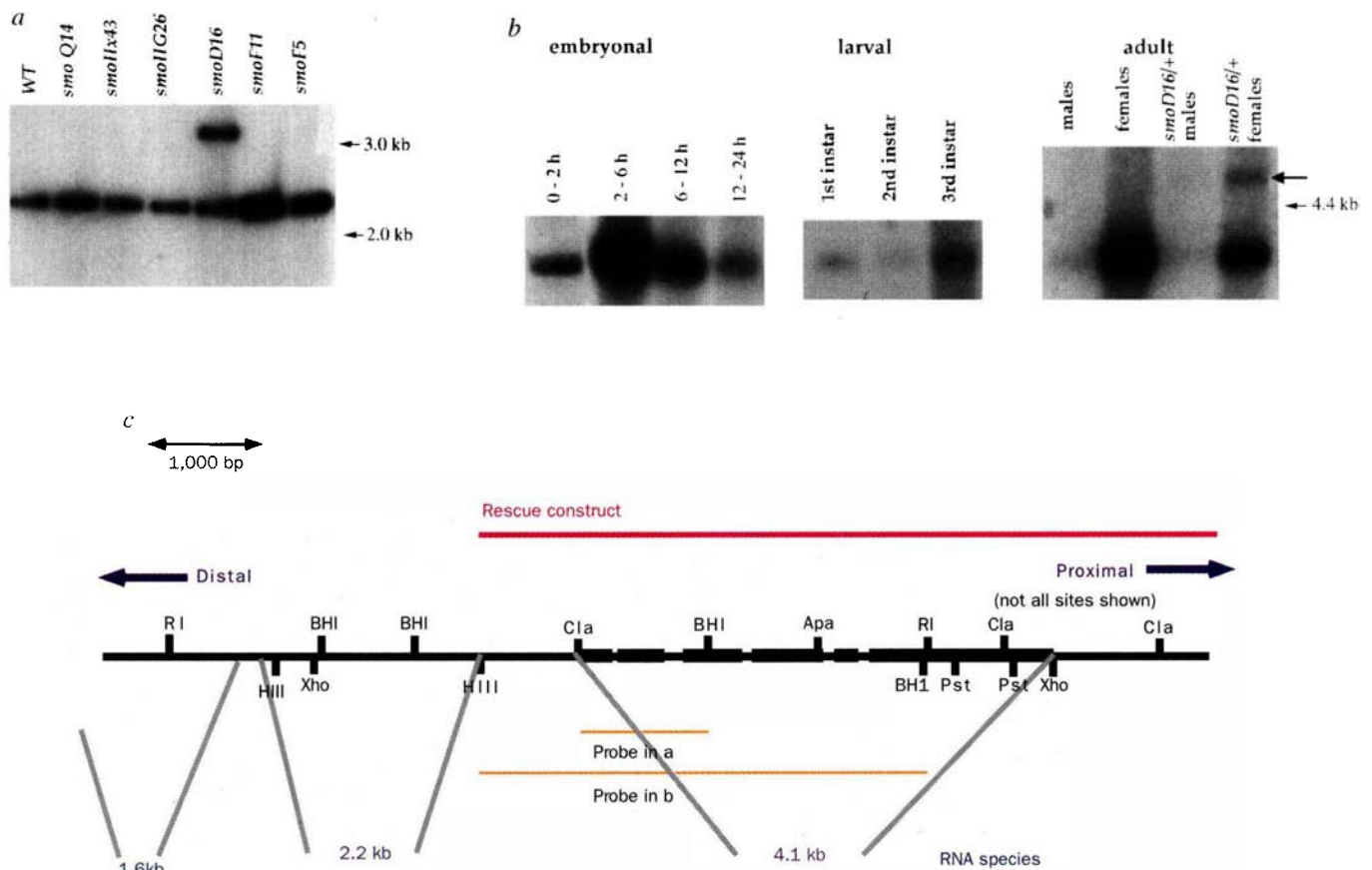


FIG. 4 a, Southern blot of genomic DNA (digested with *Bam*HI) from different *smo* alleles, hybridized with a subclone from a P1 clone (see c). Six P1 clones, covering the area of 21B7-8, the region to which *smo* maps (based on the exclusion from *Df* 2L(*al*) and *Df* 2L(*PMF*)), were screened for the detection of aberrations in *smo* alleles. b, Developmental northern blot hybridized with subclone as in c. c, Restriction map of the *smo* locus. Complementary DNAs for all three transcripts were isolated. The genomic DNA used to make a transgenic fly is indicated. Bold bars indicate the *smo* gene exons. d, The one-letter amino-acid sequence of the longest open reading frame in the *smo* gene is shown. Three more methionine codons are found in-frame before the indicated start of translation, two of which are not surrounded by residues creating a good translation start site<sup>31</sup>; a third methionine, just one codon before, is interrupted by an intron. Hydrophobic stretches are indicated in red, the first one probably representing a signal sequence. Arrow denotes a putative signal peptidase cleavage site. Putative N-linked glycosylation sites are highlighted in green. The homology with the *Drosophila* Fz protein is confined to the putative transmembrane region, except for the cysteine residues, part of a cysteine-rich domain in Fz proteins, shown in purple. Residues that could correspond to phosphorylation sites by G-protein-coupled receptor kinases and cAMP-dependent protein kinase A are highlighted in blue and grey, respectively.

**METHODS.** P1 phage DNA was prepared by the alkaline lysis method and digested; DNA fragments were isolated from agarose gels by spinning through glasswool and subcloned in BlueScript (Stratagene). Genomic fly DNA was prepared, digested with restriction enzymes, separated on agarose gels and transferred to Hybond N (Amersham). P1 fragments were labelled with [ $\alpha^{32}$ P]dCTP (Amersham protocol) and hybridized to the filters under standard conditions. Total RNA from different stages was prepared by guanidine-HCl extraction and acetic acid precipitation. RNA samples were run under identical conditions and the amount of RNA loaded was controlled by staining the blot with methylene blue after hybridization. The restriction fragment indicated in c was isolated and cloned into pCaSpeR for transformation. Transgenic flies were generated and identified among the  $F_1$  progeny on the basis of their eye pigmentation, and balanced lines were established. A partial complementary DNA for *smo* was isolated

d

|             |            |            |             |            |      |
|-------------|------------|------------|-------------|------------|------|
| MMFLEVAIRC  | LWVVDASAS  | SAKFGSTTPA | SAQQSDVELE  | PINGTLNYRL | 50   |
| YAKKGRDDPK  | WFDGLDSRHI | QCVRRCARYP | TSNATNTCFG  | SKLPYELSSL | 100  |
| DLTDFHTEKE  | LNDKLDNYA  | LKHVPKCWAA | IQPFLCAVFK  | PKCEKINGED | 150  |
| MVYLPSYEMC  | RITMEPCRI  | YNTTFPPKFL | RCNETLFPPTK | CTNGARGMKF | 200  |
| NGTGQCLSP   | VPTDTSASY  | PGIEVCGVRC | KDPLYTDDH   | ROIHKLIGWA | 250  |
| GSICLLSNLF  | VVSTFFIDWK | NANKYPAVIV | FYINLCFLIA  | CVGWLLOFTS | 300  |
| GSREDIVCRK  | DGTLRHSEPT | AGENLSCIVI | FVLVYVFLTA  | GMVWFVFLTY | 350  |
| AWHWRAMGHV  | QDRIDKKGSY | FHLVAWSLPL | VLTTTMAPFS  | EVDGNSIVGI | 400  |
| CFVGYINHSM  | RAGLLLGPLC | GVILSGGYFI | TRGMVMLFGL  | KHFANDIKST | 450  |
| SASNKIHLII  | MRMGVCALLT | LVFILVAIAC | HVTEFRHADE  | WAQSFROFII | 500  |
| CKISSVFEEK  | SSCRIENRPS | VGVLQLHLIC | LFSSGIVMST  | WCWTPSSIET | 550  |
| WKRYIRKKG   | KEVVEEVKMP | KHKVIAQTA  | KRKDFEDKGR  | LSITLYNTH  | 600  |
| DPVGLNFDVN  | DLNSSETNDI | SSTWAAVLP  | CVKRRMALTG  | AATGNSSSHG | 650  |
| PRKNSLDSEI  | SVSVRHVSVE | SRRNSVDSQV | SVKIAEMTK   | VASRSRGKHG | 700  |
| GSSSNRRTOR  | RRDYIAAATG | KSSRRRESST | SVESQVIALK  | KTTYPNASHK | 750  |
| VGVFAHHSSK  | KQHNYTSSMK | RRTANAGLDP | SILNEFLQKN  | GDFIFPFLQN | 800  |
| QDMSSSSSEED | NSRASQKIQD | LNVVVKQYFI | SEDDHDGIKI  | EELPNSKQVA | 850  |
| LENFLKNIKK  | SNESNSNRHS | RNSARSQSKK | SQKRHLKNPA  | ADLDFRKDCV | 900  |
| KYRSNDSLSC  | SSELDVALD  | VGSLLNSSFS | GISMKGPHSR  | NSKTSQDVGI | 950  |
| QANPFELVPS  | YGEDELQAM  | RLNAASRQR  | TEAANEDFGG  | TELQGLLGHS | 1000 |
| HRHQREPTFM  | SESDKLKMLL | LPSQ       |             |            | 1024 |

from a 0–2 h cDNA library<sup>32</sup> by hybridization with a probe contained within the rescue fragment. The *smo* cDNA and genomic subclones completely covering the region of the rescue fragment were sequenced using the dideoxy method with Sequenase (USB); both strands of the DNA were sequenced at least twice. The start of transcription and intron–exon boundaries were confirmed by sequencing fragments generated by RT–PCR, using primers designed from the genomic sequence.



Expression of *dpp* is lost, however, from clones of cells lacking *smo* activity when such clones are located within the normal *dpp* domain (Fig. 3b–e). Thus *smo* is required in a cell-autonomous manner for *dpp* expression. Transcription of *dpp* can be activated in the wing disc independently of *hh* by removing the activity of cAMP-dependent protein kinase (PKA), suggesting that PKA acts downstream of *hh* to antagonize its activity<sup>17</sup>. To determine whether such *hh* independent expression of *dpp* requires the activity of *smo*, we generated clones that simultaneously lack *smo* and the PKA catalytic subunit. Such clones express *dpp* at any position within the anterior compartment of the disc (Fig. 3f, g), indicating that *smo* is not absolutely required for *dpp* transcription; rather, it acts upstream of PKA to mediate activation of *dpp* by *hh*.

We mapped the *smo* locus more precisely by recombination analysis and by using small chromosomal deficiencies localized it to bands 21B7–8 on the left arm of chromosome II. Genomic P1 clones<sup>18</sup> covering this region were obtained and used to screen genomic DNA from newly isolated  $\gamma$ -ray-induced alleles (Fig. 1) of *smo* by Southern blot hybridization. We identified a fragment contained within one of the P1 clones that consistently detects a novel restriction fragment in digests from the  $\gamma$ -ray-induced allele *smo*<sup>D16</sup> (Fig. 4a). Northern blot analysis of RNA from adult females revealed three maternally expressed transcripts recognized by this fragment (Fig. 4c). The largest of these (4.1 kilobases) shows a size alternation in RNA from *smo*<sup>D16</sup> heterozygous females (Fig. 4b), suggesting that it may represent the *smo* transcript. To investigate this possibility, a genomic fragment including only this transcription unit (Fig. 4c) was transformed into flies and tested for its ability to rescue the *smo* phenotype. Animals homozygous for *smo* alleles and carrying one copy of this transgene survive to adulthood and are phenotypically wild type, confirming that this fragment includes the *smo* transcription unit and all sequences necessary for its expression (data not shown).

The complete nucleotide sequence of the *smo* transcription unit was determined by sequencing genomic and partial complementary DNA clones (Fig. 4d) to reveal the intron–exon structure shown in Fig. 4c. Conceptual translation of the sequence identified a single long open reading frame of 1,024 amino acids (Fig. 4d) preceded by a 5' untranslated leader of at least 296 nucleotides. Downstream of the translation termination codon is an untranslated region of 654 nucleotides. Hydropathy analysis of the amino-acid sequence indicates a putative signal sequence followed by a further seven hydrophobic domains, each of which is long enough to span the membrane once. These features are typical of members of the large family of G-protein-coupled receptors (GPCR); searches of the databases revealed no significant homology to any well characterized GPCR, but did reveal limited homology to members of the Frizzled (Fz) family of serpentine proteins<sup>19</sup> (Fig. 4d). In contrast to the latter, the predicted Smo protein contains a long carboxy-terminal extension, which includes consensus target sites for PKA and G-protein-coupled receptor kinases (Fig. 4d), both of which are involved in GPCR desensitization after agonist-induced activation<sup>20</sup>. On the basis of these features, Smo may act as a receptor for the Hh protein. The involvement of both PKA and a protein with characteristics of a GPCR in transduction of the Hh signal is intriguing, given the well established roles of several GPCRs in modulating adenylyl cyclase activity and hence intracellular cyclic AMP levels<sup>20</sup>. We note, however, that PKA may not be directly regulated by *hh* activity, but rather may act in a parallel pathway to antagonize the targets of *hh* signalling<sup>17</sup>. Moreover, recent studies have emphasized the diversity of pathways by which the activity of GPCR agonists can be transduced<sup>21,22</sup>. Earlier analysis has implicated another multiple membrane-spanning protein, encoded by the segment-polarity gene *patched* (*ptc*)<sup>23,24</sup>, in the reception of the Hh signal<sup>4</sup>. Although our results cast doubt on models of Ptc as the Hh receptor, we emphasize that the functional relationship between Hh, Ptc and Smo remains unclear. As Ptc has a predicted topology reminiscent of proteins involved in transport of ions and

small molecules<sup>23,24</sup>, its activity may be regulated by Smo in a manner analogous to that of G-protein-gated ion channels<sup>25</sup>. Epistasis analysis has, however, placed *ptc* upstream of *smo*<sup>13</sup>, suggesting that there is an unprecedented relationship between these two unusual proteins, the nature of which awaits further analysis. □

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## Requirement for Ku80 in growth and immunoglobulin V(D)J recombination

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THE DNA-dependent protein kinase (DNA-PK) is a mammalian serine/threonine kinase that is implicated in the repair of DNA double-strand breaks<sup>1–4</sup>, DNA replication<sup>1,5</sup>, transcription<sup>6–8</sup>, and V(D)J recombination<sup>9–12</sup>. To determine the role of the DNA-binding subunit of DNA-PK *in vivo*, we targeted *Ku80* in mice. In mutant mice, T and B lymphocyte development is arrested at early progenitor stages and there is a profound deficiency in V(D)J rearrangement. Although *Ku80*<sup>−/−</sup> mice are viable and reproduce, they are 40–60% of the size of littermate controls.

Consistent with this growth defect, fibroblasts derived from *Ku80*<sup>-/-</sup> embryos showed an early loss of proliferating cells, a prolonged doubling time, and intact cell-cycle checkpoints that prevented cells with damaged DNA from entering the cell-cycle. The unexpected growth phenotype suggests a new and important link between Ku80 and growth control.

*Ku80* was inactivated by deleting 3.4 kilobases (kb) of the mouse *Ku80* locus, including 100 base pairs (bp) of the promoter and the first two exons (Fig. 1a, b). Three independently targeted embryonic stem (ES) cell clones were used to generate *Ku80*-mutant mice. All three lines displayed an identical phenotype.

To confirm that the disruption produced a null mutation, Ku80 protein was measured by western blotting using polyclonal antisera against intact mouse Ku80. Ku80 was undetectable in *Ku80*<sup>-/-</sup> fibroblasts and *Ku80*<sup>-/-</sup> ES cells, whereas the Ku70 subunit of the Ku heterodimer was present, but at much reduced levels (Fig. 1c). The decrease in the level of Ku70 is consistent with reports that the stability of Ku70 is compromised by the absence of functional Ku80 (refs 9, 10, 13, 14). The lack of Ku80 is also reflected in the absence of Ku DNA-end-binding activity, and an increased sensitivity of *Ku80*<sup>-/-</sup> ES cells to  $\gamma$ -irradiation (A.N., manuscript in preparation)<sup>15,16</sup>.

Heterozygous *Ku80*<sup>+/-</sup> mice were indistinguishable from their wild-type littermates in all respects, and 25% of the offspring born from *Ku80*<sup>+/-</sup>  $\times$  *Ku80*<sup>+/-</sup> crosses were *Ku80*<sup>-/-</sup>. In contrast, newborn *Ku80*<sup>-/-</sup> mice were significantly smaller than their wild-type and heterozygous littermates (Fig. 2a, b). Whereas 50% of the *Ku80*<sup>-/-</sup> mice in the initial litters failed to thrive and died within 3 days of birth, survival of *Ku80*<sup>-/-</sup> mice improved to 80% when their larger *Ku80*<sup>+/-</sup> and *Ku80*<sup>+/+</sup> littermates were removed. Adult *Ku80*<sup>-/-</sup> mice were fertile, but their average litter size (4 pups) was 50% smaller than *Ku80*<sup>+/-</sup> or *Ku80*<sup>+/+</sup> mice (8 pups). In addition, *Ku80*<sup>-/-</sup> females were unable to sustain their newborn pups, which died within a few days unless they were nursed by a foster mother.

To establish when growth retardation first appeared, *Ku80*<sup>-/-</sup> embryos were isolated, examined and weighed starting at embryonic day 13.5 (e13.5). Although *Ku80* transcripts are normally detectable from the 1-cell stage and presumably function throughout development (A.N., manuscript in preparation), the size difference was first noted at e15.5, and became statistically

significant at e17.5 (Fig. 2b). This difference increased to ~40% at birth (Fig. 2b). During a 6-month observation period, *Ku80*<sup>-/-</sup> mice grew and maintained body weights at 40–60% of controls, remaining proportional dwarfs (Fig. 2c). The reduced body weight in *Ku80*<sup>-/-</sup> mice does not appear to be a consequence of cell size, because comparisons of cell dimensions in kidney, muscle and liver sections revealed no significant differences.

To characterize the growth deficiency further, we derived primary fibroblasts from mutant and wild-type embryos (MEFs) and monitored their growth *in vitro* (Figs 1c, 2d). Early-passage *Ku80*<sup>-/-</sup> MEFs grew about twice as slowly as either *Ku80*<sup>+/-</sup> or *Ku80*<sup>+/+</sup> fibroblasts (Fig. 2d). This difference in doubling time was partly due to a decrease in the number of dividing cells in *Ku80*<sup>-/-</sup> cultures, as determined by the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA during a 48-hour labelling period. Only 75% of the passage two *Ku80*<sup>-/-</sup> MEFs were actively cycling, compared to 94% for the controls. Of the non-cycling *Ku80*<sup>-/-</sup> cells, 81% were in G1 and 19% were in G2/M, whereas the majority (99%) of the non-cycling controls were arrested in G1. Proliferation decreased with passage in culture, and by passages 4–6, fibroblasts from *Ku80*<sup>-/-</sup> mice failed to divide. Passage-4 *Ku80*<sup>-/-</sup> MEFs appeared to be larger than controls, and the monolayers contained giant polyploid cells, suggesting premature entry into senescence (Fig. 3a, bottom). The early appearance of non-dividing cells in preparations of *Ku80*<sup>-/-</sup> MEFs is consistent with the correlation between senescence and defective DNA repair<sup>17</sup>.

To analyse the proliferating population, we pulse-labelled cells with BrdU for 2 hours, and then followed their progression through the cell cycle (Fig. 2e). Consistent with the continuous labelling experiment, we found that during a 2-hour pulse, 33% fewer *Ku80*<sup>-/-</sup> cells incorporated BrdU compared to controls. In the cycling population, the relative distribution of cells in S (60%) and G2/M (38%) phases was similar in *Ku80*<sup>-/-</sup> MEFs and controls immediately after the pulse (Fig. 2e). During the chase period, labelled *Ku80*<sup>-/-</sup> MEFs accumulated in G2/M and exhibited a delay in progression through G2/M into G1. After a 2-hour chase, 59% of the cycling *Ku80*<sup>-/-</sup> MEFs were still in G2/M, and only 10% had moved into G1, whereas 39% of the controls were in G2/M and 22% had already completed mitosis (Fig. 2e). When

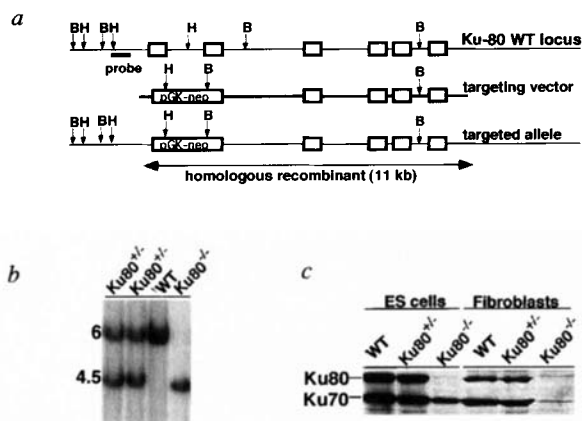


FIG. 1 Inactivation of *Ku80* by homologous recombination. a, Scheme of the *Ku80* gene and hybridization probe (top), targeting construct (middle), and the target allele (bottom) (not to scale). *Bgl*II and *Hind*III restriction sites used to detect the targeted gene are indicated. b, Southern blot of *Bgl*II-digested tail DNA from control (+/+), heterozygous (+/-) and homozygous (-/-) *Ku80*-targeted mice. The wild-type and mutant fragments are 6 and 4.5 kb respectively. c, Ku80 protein is not expressed in *Ku80*<sup>-/-</sup> cells. Whole-cell lysates prepared from ES cells (50  $\mu$ g) and mouse embryo fibroblasts (MEFs, 100  $\mu$ g) were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a mixture of polyclonal antibodies against full-length rodent Ku80 (1:5,000 dilution)

and Ku70 (1:5,000 dilution). Staining was with enhanced chemiluminescent detection reagents (Amersham). The 90K band seen in *Ku80*<sup>-/-</sup> lanes is a background band that runs above authentic Ku80. Abbreviations: B, *Bgl*II site; H, *Hind*III site; pGK-neo, neomycin-resistance gene driven by the pGK promoter; *Ku80*<sup>+/-</sup>, heterozygous *Ku80* mutant; *Ku80*<sup>-/-</sup>, homozygous *Ku80* mutant; WT, wild type; fibroblasts, mouse embryonic fibroblasts (MEFs).

**METHODS.** Mouse *Ku80* was isolated from a 129SVJ strain genomic library (Stratagene). The replacement vector was constructed using a 1.2-kb fragment 5' of the translation start site, and a 9-kb *Bgl*II–*Eco*RI fragment extending into R1 embryonic stem cells and selection in 200  $\mu$ g ml<sup>-1</sup> G418, 11 of 176 (6%) neomycin-resistant clones were found to be homologous recombinants by Southern blotting. Six clones were injected into C57B1/6 blastocysts, and three of them produced chimaeric mice that transmitted the mutation. The *Ku80*<sup>-/-</sup> ES cell line was generated from *Ku80*<sup>+/-</sup> ES cells by selection in 1 mg ml<sup>-1</sup> G418. MEFs were derived from e13.5 embryos obtained by intercrossing *Ku80*<sup>+/-</sup> mice. The genotype of the embryos was determined by PCR which distinguishes endogenous from the targeted *Ku80* allele. The PCR reactions contained 100 ng genomic DNA; 0.5  $\mu$ M (each) of primers K1: ATTGTGATGTGTGGACACG, K2: AGCTTC-CACCTCTAGAGAT and K3: TAAAGCGCATGCTCCAGACT; 100 mM (each) dNTP; 1.5 mM MgCl<sub>2</sub> and 0.25 U of *Taq* polymerase. Cycling conditions were 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (30 cycles), followed by an extension at 72 °C for 10 min. Primers K1 and K2 give a product diagnostic of the targeted allele that is ~200 bp; primers K1 and K3 yield a wild-type product of ~350 bp. Cell lysates were prepared as described<sup>8</sup>. Anti-Ku80 and Anti-Ku70 polyclonal antibodies were generated in rabbits using full-length recombinant mouse Ku80 and rat Ku70 as antigens.

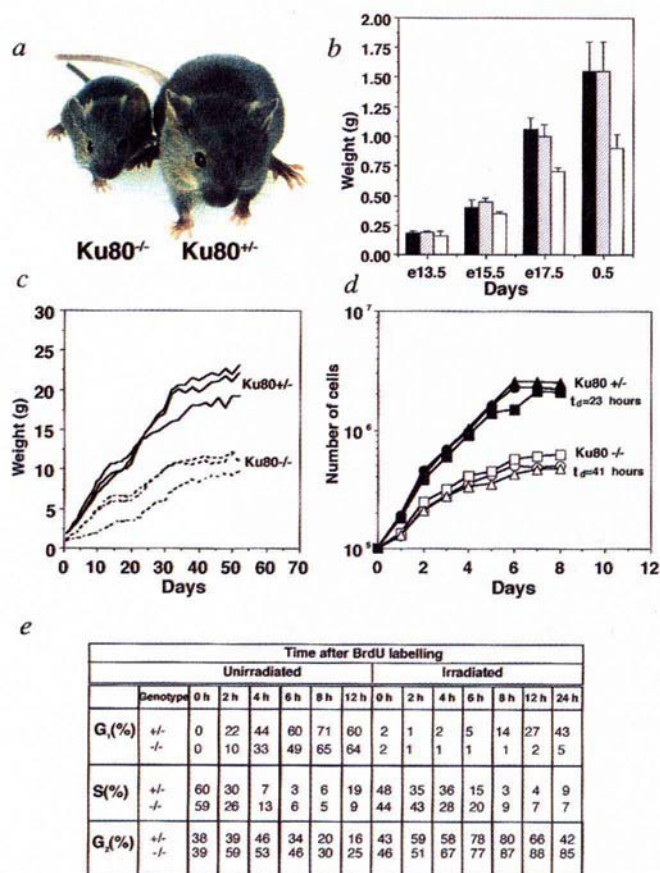


FIG. 2 *Ku80* deficiency alters both pre- and postnatal growth. **a**, Photograph of 3-week-old *Ku80*<sup>-/-</sup> and *Ku80*<sup>+/-</sup> littermates (there is no significant difference in size between *Ku80*<sup>+/-</sup> and *Ku80*<sup>-/-</sup> mice; not shown). **b**, Growth of *Ku80*<sup>-/-</sup> and *Ku80*<sup>+/-</sup> embryos, as measured by weight. Pregnant females from *Ku80*<sup>-/-</sup> intercrosses were killed at e13.5, e15.5 or e17.5 and 5–10 embryos of each genotype were weighed. The average weights of wild-type (filled bars), heterozygous (hatched bars) and homozygous (white bars) embryos and of newborn mice, are plotted. Error bars represent the standard deviation from the mean for each genotype. Statistical analysis using Student's *t*-test ( $P < 0.05$ ) showed significant differences in mean values between *Ku80*<sup>-/-</sup> versus *Ku80*<sup>+/-</sup> and *Ku80*<sup>+/-</sup>, starting at e17.5. **c**, Postnatal growth of three female *Ku80*<sup>+/-</sup> and three *Ku80*<sup>-/-</sup> littermates. Weights of individual animals are plotted against time. **d**, Growth kinetics of primary fibroblasts.  $10^5$  passage-two MEFs were plated in replicate 60-mm dishes and individual dishes were trypsinized and counted every 24 h as indicated. Doubling time was calculated to be 41 h for *Ku80*<sup>-/-</sup>, compared to 23 h for *Ku80*<sup>+/-</sup>, based on the exponential portion of the growth curve. *Ku80*<sup>-/-</sup> MEFs reached a 4-fold lower saturation density than controls ( $2.66 \times 10^4$  per cm<sup>2</sup> for *Ku80*<sup>-/-</sup>, compared to  $1.1 \times 10^5$  per cm<sup>2</sup> for *Ku80*<sup>+/-</sup>). Wild-type and *Ku80*<sup>+/-</sup> MEFs were indistinguishable (not shown). **e**, Cell-cycle progression of S-phase *Ku80*<sup>+/-</sup> (+/-) and *Ku80*<sup>-/-</sup> (-/-) MEFs after a 2-h pulse with BrdU and a 10-Gy dose of  $\gamma$ -irradiation. The percentage of G<sub>1</sub>, S and G<sub>2</sub>/M-phase cells among the cycling population (BrdU-positive) is quantified. Data are from one of two experiments that gave similar results.

**METHODS.** e13.5 MEFs (Fig. 1) were grown in DMEM, supplemented with 10% FCS and antibiotics, and replenished daily with fresh medium. For cell-cycle analysis, exponentially growing passage-2 MEFs were labelled with 10  $\mu$ M BrdU (Sigma) at 37 °C for 2 h and then mock-treated or irradiated with 10 Gy. After the chase period, cells were fixed in 75% ethanol, stained with anti-BrdU antibody, and then counterstained with 5  $\mu$ g ml<sup>-1</sup> propidium iodide. Bivariate distributions of BrdU and propidium iodide were measured with a FACSstar system (Becton-Dickinson); at least 10,000 cells were analysed per data point.

compared to controls, a greater fraction of *Ku80*<sup>-/-</sup> cells were found in G<sub>2</sub>/M throughout the chase period, and fewer mutant cells re-entered S phase by 12 hours.

Following treatment with 10 Gy of  $\gamma$ -irradiation, both control and *Ku80*<sup>-/-</sup> cells exhibited a radiation-induced delay in S phase before arresting in G<sub>2</sub>/M (Fig. 2e). However, control cells recovered from the initial block and entered G<sub>1</sub> by 8 hours post-irradiation, whereas *Ku80*<sup>-/-</sup> cells failed to complete the cell cycle and remained in G<sub>2</sub>/M, even after 24 hours. Similarly, we found that the G<sub>1</sub> checkpoint was intact for *Ku80*<sup>-/-</sup> MEFs; serum-starved cells were irradiated with 20 Gy and then stimulated to enter the cell cycle with complete medium containing BrdU. Twenty-four hours after irradiation, 25% of control cells had re-entered the cell cycle. By contrast, *Ku80*<sup>-/-</sup> cells failed to re-enter S phase from G<sub>1</sub> after irradiation. Without irradiation, 71% of control and only 25% of *Ku80*<sup>-/-</sup> MEFs were labelled with BrdU after release from serum starvation. Our data indicate that in the absence of Ku80, cells sustaining DNA damage during either G<sub>1</sub> or G<sub>2</sub>/M fail to resume the cell cycle.

To determine whether there were specific pathological changes in the target mice, we examined histological sections from various organs. With the exception of lymphoid organs, *Ku80*<sup>-/-</sup> mice appeared normal, and their organ weights were proportional to the total body weight. However, spleen and lymph nodes were 5–10 fold smaller than controls and were devoid of lymphocytes (Fig. 3a). The thymus was also disproportionately small and had no cortical-medullary boundaries (Fig. 3a). In contrast to spleen and lymph nodes, the thymus did contain lymphoid cells, but the number of thymocytes was 10–100-fold less than in the controls ( $1\text{--}7 \times 10^6$  in *Ku80*<sup>-/-</sup> mice, compared to  $0.7\text{--}3 \times 10^8$  in controls, measured in 10 mice of each genotype).

To characterize further the immunological defect in *Ku80*<sup>-/-</sup> mice, cells from spleen, bone marrow and thymus were analysed using monoclonal antibodies specific for lymphocyte surface

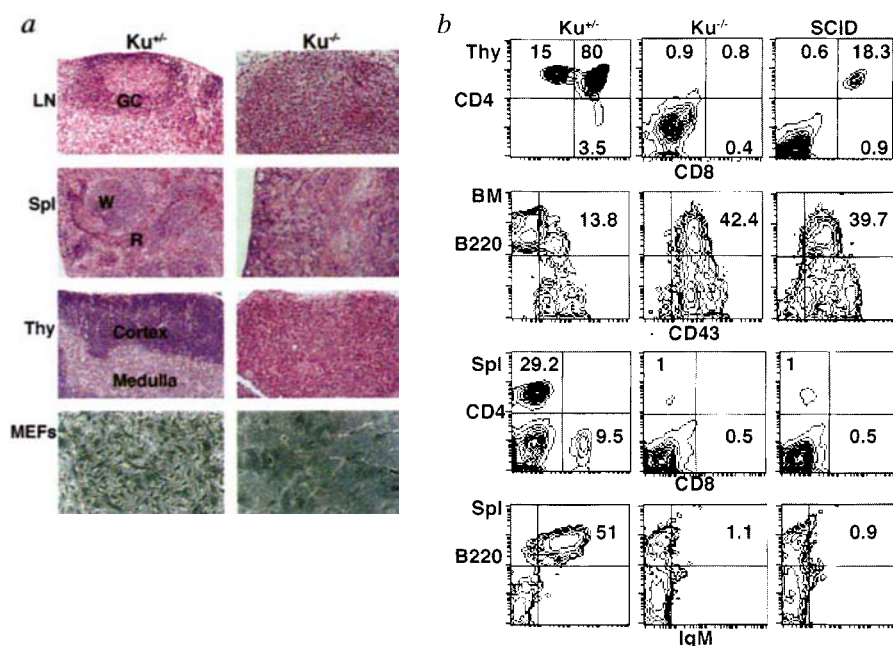
markers. Consistent with the histology, there was a complete absence of mature T and B cells in the spleen (Fig. 3b, bottom). Examination of the bone marrow and thymus showed that both T- and B-cell development was blocked at early precursor stages (Fig. 3b, top). B cells were arrested at the B220<sup>+</sup>CD43<sup>+</sup> stage, and only CD4<sup>-</sup>CD8<sup>-</sup> cells were found in the thymus. The early restriction of lymphocyte maturation in the *Ku80*<sup>-/-</sup> thymus was clearly different from SCID controls, which displayed more mature CD4<sup>+</sup>CD8<sup>+</sup> cells. Thus the immunological phenotype in *Ku80*<sup>-/-</sup> mice more closely resembles that found in *RAG*<sup>-/-</sup> mice<sup>18,19</sup> and is distinct from SCID, where the underlying genetic defect is in the catalytic subunit of DNA-PK (DNA-PK<sub>c</sub>)<sup>11,12,20</sup>.

Transient transfection experiments in mutant cell lines have shown that *Ku80* is required for the formation of coding and signal joints during V(D)J recombination<sup>9,10</sup>. To determine whether the *Ku80*<sup>-/-</sup> mutation affects rearrangements of antigen-receptor gene segments in T and B lymphocytes *in vivo*, we measured immunoglobulin and T-cell antigen receptor (TCR) rearrangements by polymerase chain reaction (PCR)<sup>21–24</sup>. Figure 4a shows that *Ku80*<sup>-/-</sup> T cells do undergo D $\delta$ 2–J $\delta$ 1 recombination, but at a level somewhat lower than that found in SCID. When normalized to the internal control germline band (~1 kb) there were on average ( $n = 8$ ) 2–3-fold fewer D $\delta$ 2–J $\delta$ 1 joints in *Ku80*<sup>-/-</sup> than in SCID thymocytes. Similarly, DJ<sub>H</sub> rearrangements were found in *Ku80*<sup>-/-</sup> bone-marrow cells, but at a lower incidence than in SCID (Fig. 4c). A more striking difference between *Ku80*<sup>-/-</sup> and SCID mice was seen in their ability to generate signal joints (Fig. 4a). Whereas D $\delta$ 1–D $\delta$ 2 signal junctions were present in both SCID and wild-type thymocytes, they were undetectable in *Ku80*<sup>-/-</sup> thymocytes (Fig. 4a). Likewise, signal ends were not found in *Ku80*<sup>-/-</sup> thymus DNA by ligation-mediated PCR<sup>22</sup> (Fig. 4b). The lack of V to DJ rearrangements at either immunoglobulin or TCR loci in *Ku80*<sup>-/-</sup> mice (Fig. 4a, c) may account for the absence of mature lymphocytes<sup>18,19</sup>. These results show that low levels of DJ



FIG. 3 Lymphocyte development is blocked at an early stage in *Ku80*<sup>-/-</sup> mice. **a**, Histology of thymus, lymph nodes, spleens and MEFs from *Ku80*<sup>-/-</sup> mice and littermate controls. **b**, Flow-cytometric analysis of thymus, bone marrow, and spleen cells from *Ku80*<sup>-/-</sup> mice, *Ku80*<sup>+/-</sup> littermates, and SCID controls. LN, lymph node; Spl, Spleen; Thy, thymus (cortex, medulla indicated); MEFs, mouse embryonic fibroblasts; GC, germinal centre; W, white pulp; R, red pulp; CD4, anti-CD4 monoclonal antibody; CD8, anti-CD8 monoclonal antibody; B220, anti-B220 monoclonal antibody; CD43, anti-CD43 monoclonal antibody; IgM, anti-IgM heavy-chain monoclonal antibody.

**METHOD.** Lymph nodes, spleens and thymuses were photographed originally at 10× magnification. Tissues from 5-week-old mice were fixed with formalin and tissue sections stained with haematoxylin and eosin. MEFs were cultured for four passages, fixed with 70% ethanol and photographed at 10× magnification. For flow-cytometry, single-cell suspensions were prepared from lymphoid organs of 4–6-week-old mice, and analysed on a Becton-Dickinson FACScan with CELLQuest software. Cells were stained with combinations of phycoerythrin (PE) labelled anti-CD4, and fluorescein (FITC)-labelled anti-CD8, or PE labelled



anti-B220, and FITC-labelled anti-CD43, or FITC anti- $\mu$  and PE anti-B220 (Pharmingen), as indicated.

coding junctions can be found in the absence of Ku80, but that efficient completion of VDJ recombination requires Ku80.

The DNA-PK $\kappa$  defect in SCID mice is not associated with growth retardation, and the immunodeficiency in SCID is less severe than in *Ku80*<sup>-/-</sup> mice. There are several explanations for the finding that mutations in two different components of the DNA-PK complex result in discrete phenotypes. First, Ku may have functions that are independent of its association with DNA-PK $\kappa$ . For example, Ku helps maintain normal telomere lengths in

*Saccharomyces cerevisiae*<sup>25</sup>. In addition, the absence of Ku80 results in a concomitant loss of DNA-PK $\kappa$  activity<sup>26</sup>, thereby compounding the primary defect. By contrast, DNA-PK $\kappa$  is not required for Ku assembly, and Ku-dependent DNA binding is normal in SCID mice<sup>3,27</sup>. Thus, broken DNA ends should still be protected by Ku in SCID mice, increasing the probability of recombination or repair. In addition, the nature of the DNA-PK $\kappa$  mutation in SCID has not been determined, but appears to involve only a partial loss of function<sup>11,12</sup>. Therefore SCID mice

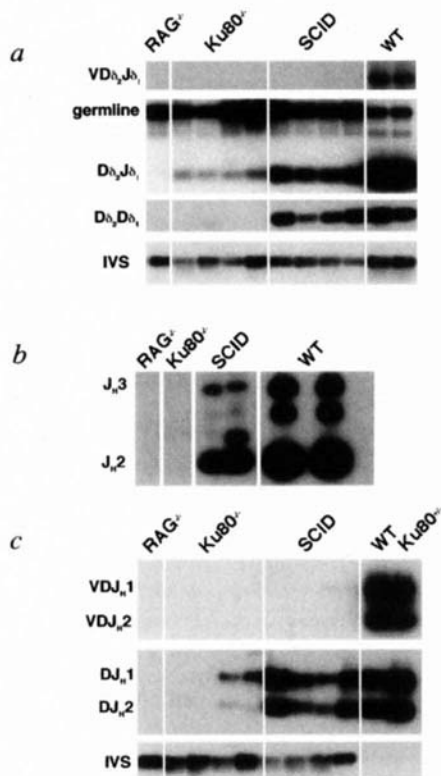


FIG. 4 T-cell antigen receptor and immunoglobulin gene rearrangement in *Ku80*<sup>-/-</sup> mice. **a**, PCR analysis of TCR gene rearrangements. Thymus DNA was assayed for recombination of V $\delta_1$  to D $\delta_2$ J $\delta_1$  (VD $\delta_2$ J $\delta_1$ ), D $\delta_2$  to J $\delta_1$  (D $\delta_2$ J $\delta_1$ ), and circular excision products D $\delta_1$ -D $\delta_2$  (D $\delta_2$ D $\delta_1$ ). Samples were from 4–6-week-old mice, with the exception of the wild type, which was from a newborn mouse. Controls include a 1-kb germline interval amplified in the D $\delta_2$  to J $\delta_1$  intervening region (germline), and a non-recombining segment of the Ig locus between J $\delta_1$  and CH1 (IVS)<sup>21</sup>. **b**, Absence of double-strand signal sequence breaks in *Ku80*<sup>-/-</sup> mice. Thymus DNA was assayed for double-strand 5'-phosphorylated J $\delta_2$  and J $\delta_3$ . Three mice of each genotype have been assayed with identical results. Each linker-ligated DNA sample was amplified using control primers from the J $\delta_1$ -CH1 non-recombining segment to ensure that similar amounts of DNA was used for LM-PCR (as in **a**). **c**, Recombination of VH558L to DJ $\delta_1$  and DJ $\delta_2$  to J $\delta_1$  gene segments<sup>21</sup>. 100 ng DNA was used for RAG<sup>-/-</sup>, *Ku80*<sup>-/-</sup> and SCID mice, and only 10 ng for *Ku80*<sup>+/-</sup> and *Ku80*<sup>-/-</sup> mice. For IVS controls, DNA was diluted 10-fold before PCR, accounting for the lack of IVS signal for *Ku80*<sup>+/-</sup> and *Ku80*<sup>-/-</sup> mice. Abbreviations: VD $\delta_2$ J $\delta_1$ , V $\delta_1$  to D $\delta_2$ J $\delta_1$  rearrangements; germline, unrecombined DNA from the D $\delta_2$  to J $\delta_1$  interval; D $\delta_2$ J $\delta_1$ , D $\delta_2$  to J $\delta_1$  rearrangement; D $\delta_2$ D $\delta_1$ , the circular excision products of D $\delta_1$ -D $\delta_2$  rearrangements; IVS, non-recombining segment of the Ig locus between J $\delta_1$  and CH1 (ref. 21); J $\delta_3$ , J $\delta_3$  signal end; J $\delta_2$ , J $\delta_2$  signal end; VD $\delta_2$ J $\delta_1$ , VH558L to DJ $\delta_1$  rearrangement; VD $\delta_2$ J $\delta_2$ , VH558L to DJ $\delta_2$  rearrangement.

**METHODS.** Genomic DNA from thymocytes was prepared by embedding  $\sim 3 \times 10^5$  cells in an agarose matrix, followed by proteinase-K digestion. DNA from  $\sim 3 \times 10^4$  thymocytes was used for PCR (**a**, **b**). D $\delta_2$ -J $\delta_1$  (ref. 29), V $\delta_1$  to D $\delta_2$ -J $\delta_1$  (ref. 23) and excised D $\delta_1$ -D $\delta_2$  signal junctions<sup>24</sup> were amplified and visualized as described<sup>23,24,29</sup>. Bone marrow DNA from 4–6-week-old mice was prepared and assayed for V $\delta_1$  to DJ $\delta_1$  and DJ $\delta_2$  to J $\delta_1$  rearrangements as described<sup>21</sup>. Each lane represents an independently derived DNA sample from an individual mouse, and all experiments were repeated at least 3 times.



may not exhibit the full range of phenotypic anomalies expected in a DNA-PK<sub>cs</sub> null mutation.

The ability of DNA-PK to phosphorylate substrates implicated in the regulation of the cell cycle<sup>5</sup>, and the sequence homology of the catalytic subunit of DNA-PK to the phosphatidylinositol-3-OH kinase family and the ataxia telangiectasia gene product<sup>28</sup>, support a role for DNA-PK in signal transduction and cell-cycle control. The fact that DNA damage in *Ku80*<sup>-/-</sup> MEFs induces delays in cell-cycle progression suggest that Ku80 is not required for recognizing DNA lesions, nor for transmitting signals that couple DNA damage to cell-cycle arrest. Consistent with the preservation of cell-cycle checkpoints, Ku80-deficient mice are not predisposed to early tumorigenesis (our unpublished observations). On the other hand, the presence of unrepaired DNA breaks that arise during normal cellular metabolism or during VDJ recombination might prevent *Ku80*<sup>-/-</sup> cells from resuming the cell cycle. Thus, arrested lymphocyte development and proportional dwarfism in *Ku80*<sup>-/-</sup> mice may be the result of abnormal DNA metabolism and a subsequent loss of cells with damaged or incomplete genomes. In conclusion, our findings provide new insights into the physiological function of Ku80, and a model to study DNA double-strand-break repair *in vivo*. □

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## Structural basis for inhibition of receptor protein-tyrosine phosphatase- $\alpha$ by dimerization

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RECEPTOR-LIKE protein-tyrosine phosphatases (RPTPs), like their non-receptor counterparts, regulate the level of phosphotyrosine-containing proteins derived from the action of protein-tyrosine kinases<sup>1</sup>. RPTPs are type-I integral membrane proteins which contain one or two catalytic domains in their cytoplasmic region<sup>2</sup>. It is not known whether extracellular ligands regulate the activity of RPTPs. Here we describe the crystal structure of the membrane-proximal catalytic domain (D1) of a typical RPTP, murine RPTP $\alpha$ . Significant structural deviations from the PTP1B fold reside within the amino-terminal helix–turn–helix segment of RPTP $\alpha$ D1 (residues 214 to 242) and a distinctive two-stranded  $\beta$ -sheet formed between residues 211–213 and 458–461. The turn of the N-terminal segment inserts into the active site of a dyad-related D1 monomer. On the basis of two independent crystal structures, sequence alignments, and the reported biological activity of EGF receptor/CD45 chimaeras<sup>3</sup>, we propose that dimerization and active-site blockage is a physiologically important mechanism for downregulating the catalytic activity of RPTP $\alpha$  and other RPTPs.

All RPTPs share a modular primary structure that includes widely varying extracellular domains likely to bind ligands<sup>4</sup>, a single membrane-spanning segment, and one or two intracellular catalytic domains termed D1 and D2. The first catalytic domain of

RPTP $\alpha$ , D1, was expressed as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli*<sup>5</sup>, purified and cleaved with trypsin to generate a fragment containing residues 202–503. Crystals of this fragment were obtained in three different space groups (*C*222<sub>1</sub>, *P*2<sub>1</sub> and *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>). The RPTP $\alpha$ D1 structure was solved by molecular replacement in space group *C*222<sub>1</sub> and refined to 2.3 Å resolution in the related space group *P*2<sub>1</sub>. Refinement to 1.7 Å resolution in space group *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> is in progress (Table 1). The identical RPTP $\alpha$ D1 dimer (Fig. 1) is observed in the three space groups despite considerable differences in the macromolecular packing between space groups *P*2<sub>1</sub> (44% solvent) and *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (58% solvent), indicating that this oligomeric state is structurally relevant. The root-mean-square deviation between the C $\alpha$  positions of the two dimers found in the *P*2<sub>1</sub> and *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space groups is 0.6 Å. At the dyad-related interface of the RPTP $\alpha$ D1 dimer, the buried surface area measures 1,500 Å<sup>2</sup>, a value comparable to that seen with other higher affinity protein–protein interfaces<sup>6</sup>. Oligomerization occurs around a two-fold rotational axis that fixes both N-terminal extensions on the same surface of each monomer, presumably facing the plasma membrane (see Fig. 1a).

Using dynamic light scattering<sup>7</sup> (data not shown) and gel filtration chromatography at RPTP $\alpha$ D1 concentrations of 0.1 to 5.0 mg ml<sup>-1</sup>, we observe monomers, dimers and higher oligomers of RPTP $\alpha$ D1. In similar experiments with RPTP $\alpha$ D2 (residues 504–793), we only observe monomeric species. We speculate that the dimer and higher oligomers observed in solution and in the two independent crystal forms partly replicate the oligomerization of intact RPTP $\alpha$  in the membrane. The increase in local concentration of RPTP $\alpha$  in the membrane, caused by the binding of multivalent ligands to extracellular domains, would significantly increase oligomerization. The additional spatial constraint that the N terminus of each RPTP $\alpha$ D1 monomer is anchored in the membrane (Fig. 1a) would enhance the dimerization potential of RPTP $\alpha$ D1 *in vivo* by fixing the orientation of the D1 monomer and restricting the diffusion of RPTP $\alpha$  to two dimensions.

Whereas the overall fold of RPTP $\alpha$ D1 closely resembles the fold previously observed for PTP1B (ref. 8) and one *Yersinia* protein-tyrosine phosphatase (PTP), Yop51 (ref. 9), the quaternary organization of the RPTP $\alpha$ D1 monomers in two independent

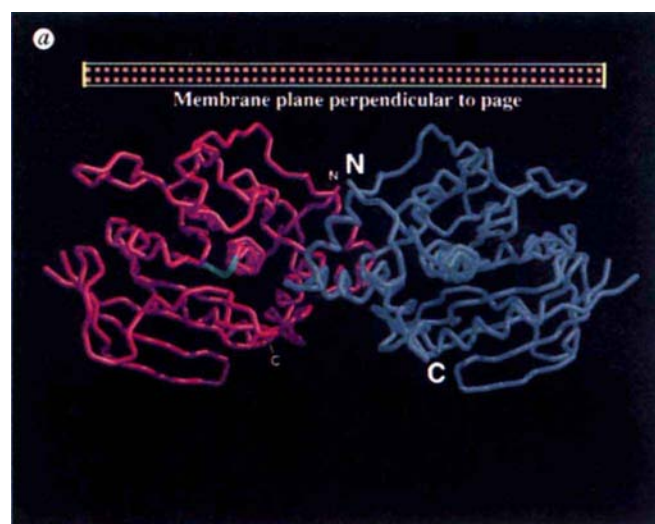
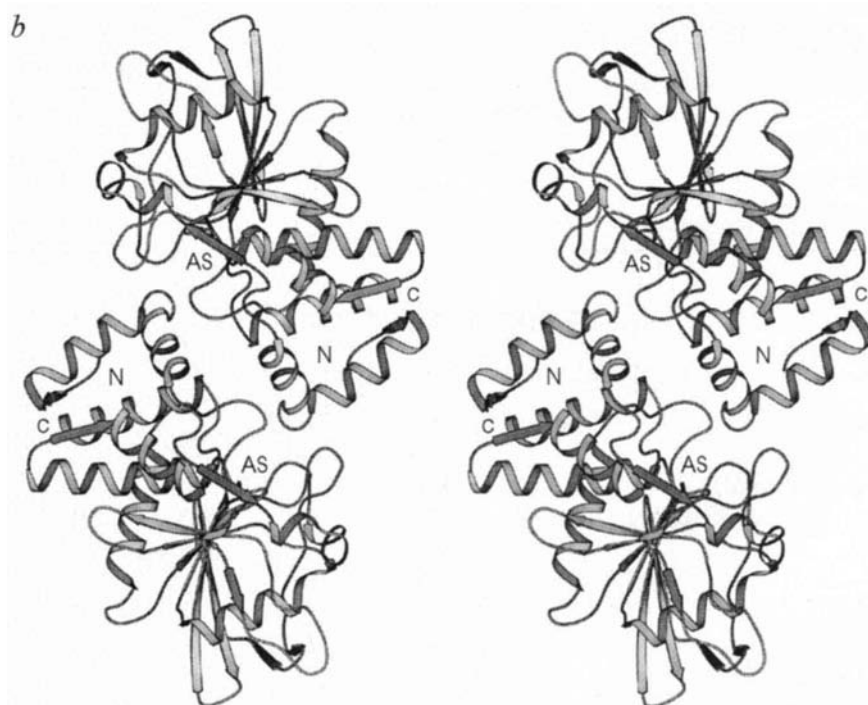


FIG. 1 Global view of the RPTP $\alpha$ D1 dimer. *a*, C $\alpha$  trace of the RPTP $\alpha$ D1 dimer. The dyad axis is oriented vertically in the plane of the figure. The N and C termini are labelled for clarity. The active-site cysteine of the pink monomer is highlighted in green. A possible orientation of the catalytic domains with respect to the plasma membrane is emphasized schematically with the orange squares boxed in yellow. This orientation with an additional 41 N-terminal residues would link D1 to the transmembrane helix and thus the extracellular domain. *b*, Stereo ribbon diagram of the RPTP $\alpha$ D1 dimer. The dyad axis has been rotated 90° towards the reader from the orientation shown in *a*. The C termini now extend away from the reader. The label AS placed near the catalytically essential Cys 433 emphasizes the active site of each monomer. Both of these views accurately represent the dimeric form of RPTP $\alpha$ D1 observed in two independent crystal forms. *a* was produced with the conic option<sup>24</sup> of MIDAS<sup>25</sup>, and *b* with SETOR<sup>26</sup>.



crystal forms is unique. In the RPTP $\alpha$ D1 dimer, the amino-terminal segment of each monomer forms a helix–turn–helix structural wedge (interhelical angle  $\sim 80^\circ$ ) tucked into the active site of the opposing monomer (Fig. 2*a, b*). Four loops, 258–264 (L1), 330–340 (L6), 398–404 (L13 or WpD) and 472–482 (L17) delineate the entrance to the active site defined by catalytically essential Cys 433 (refs 8, 10). There are hydrogen bonds and van der Waals interactions between the residues from the N-terminal wedge of one monomer and residues from the L1 and L13 (WpD) loops of the dyad-related monomer (Fig. 2*b*). These interactions restrain the mobile L13 or WpD loop of each monomer in the ‘open’ position<sup>11</sup>. In its present conformation, the structural wedge precludes the large movement of L13 (WpD) towards the active-site cysteine as observed in both a PTP1B–peptide complex<sup>11</sup> and the Yop51–sulphate complex<sup>10</sup>. In a least-squares structural superimposition of the RPTP $\alpha$ D1 monomer with the sulphate-bound form of Yop51 (Brookhaven Protein Data Bank code 1YTS), the backbone atoms of the closed WpD (L13) loop of Yop51 clash with the Asp–Asp motif (residues 227–228) of the N-terminal wedge of the dyad-related RPTP $\alpha$ D1 monomer (Fig. 2*c*). In the RPTP $\alpha$ D1 dimer, both the putative general acid, Asp 401

(ref. 12), and the phosphotyrosyl-binding group, Phe 402 (ref. 11), are firmly fixed on L13 and directed away from the active site (Fig. 2*b, c*). In addition, several side chains from the second helix of this N-terminal wedge interact with Tyr 262 and Asn 264 on L1 in the dyad-related monomer. In the PTP1B–peptide complex<sup>11</sup>, the two equivalent residues, Tyr 46 and Asp 48, form critical interactions with the phosphotyrosine-containing peptide. This stereochemical arrangement indicates that the dimeric form of RPTP $\alpha$ D1 described here is unable to bind phosphotyrosine-containing substrates and is therefore catalytically inactive. Within one monomer, the highly conserved proline-rich sequence Pro–Pro–Leu–Pro (210–213) preceding the N-terminal helix participates with residues 458–461 in three inter-strand hydrogen bonds (Fig. 3*a*). We believe that this latter structural link, together with the rigidity conferred by three proline residues, constrain the conformation of the N-terminal wedge.

The unique structural arrangement of the amino-terminal wedge and residues 458–461 of dimeric RPTP $\alpha$ D1 contrasts sharply with the corresponding segments in the non-receptor PTP1B, which are unrelated in sequence (Fig. 3). Compared with the human PTP1B primary sequence, RPTP $\alpha$ D1 contains a

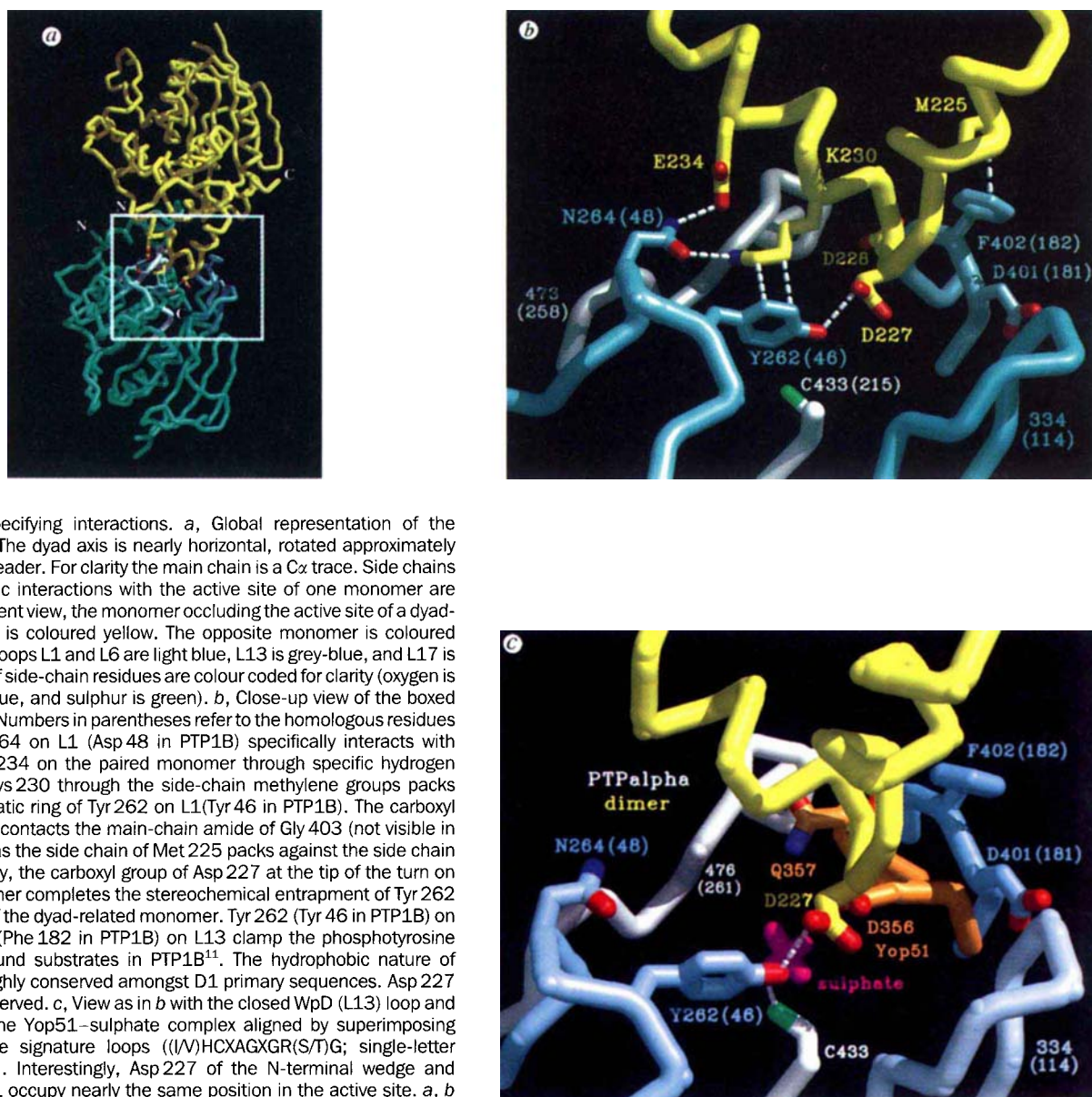


FIG. 2 Dimer specifying interactions. *a*, Global representation of the RPTP $\alpha$ D1 dimer. The dyad axis is nearly horizontal, rotated approximately 20° towards the reader. For clarity the main chain is a C $\alpha$  trace. Side chains involved in dimeric interactions with the active site of one monomer are shown. In the current view, the monomer occluding the active site of a dyad-related monomer is coloured yellow. The opposite monomer is coloured aqua. Active-site loops L1 and L6 are light blue, L13 is grey-blue, and L17 is grey. The atoms of side-chain residues are colour coded for clarity (oxygen is red, nitrogen is blue, and sulphur is green). *b*, Close-up view of the boxed region in panel *a*. Numbers in parentheses refer to the homologous residues in PTP1B<sup>8</sup>. Asn 264 on L1 (Asp 48 in PTP1B) specifically interacts with Lys 230 and Glu 234 on the paired monomer through specific hydrogen bonds. In turn, Lys 230 through the side-chain methylene groups packs against the aromatic ring of Tyr 262 on L1 (Tyr 46 in PTP1B). The carboxyl group of Asp 228 contacts the main-chain amide of Gly 403 (not visible in the figure) whereas the side chain of Met 225 packs against the side chain of Phe 402. Finally, the carboxyl group of Asp 227 at the tip of the turn on the paired monomer completes the stereochemical entrapment of Tyr 262 and the L1 loop of the dyad-related monomer. Tyr 262 (Tyr 46 in PTP1B) on L1 and Phe 402 (Phe 182 in PTP1B) on L13 clamp the phosphotyrosine side chain of bound substrates in PTP1B<sup>11</sup>. The hydrophobic nature of residue 230 is highly conserved amongst D1 primary sequences. Asp 227 also is highly conserved. *c*, View as in *b* with the closed WpD (L13) loop and sulphate ion of the Yop51-sulphate complex aligned by superimposing their phosphatase signature loops ((I/V)HCXAGXGR(S/T)G; single-letter amino-acid code). Interestingly, Asp 227 of the N-terminal wedge and Asp 356 of Yop51 occupy nearly the same position in the active site. *a*, *b* and *c* were produced with the conic option<sup>24</sup> of MIDAS<sup>25</sup>.

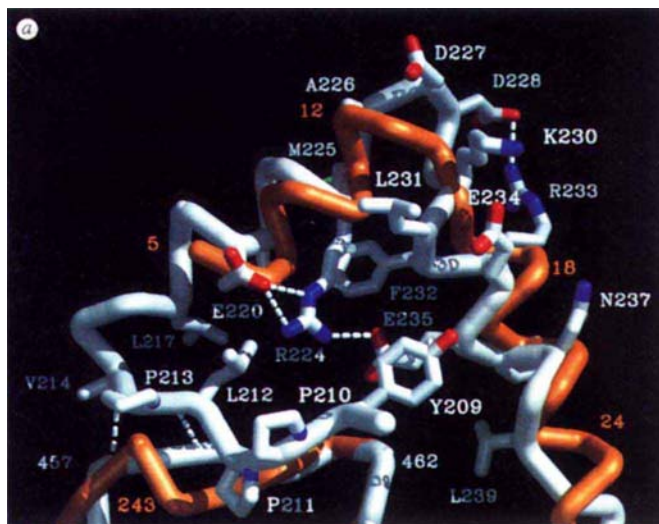
two-residue insertion in the turn linking the first and second N-terminal helices,  $\alpha 1'$  and  $\alpha 2'$  (Fig. 3*b*). Both of the inserted residues, Asp 227 and Asp 228, participate in interactions across the dimer interface of RPTP $\alpha$ D1 (Fig. 2*b*). The two-stranded  $\beta$ -sheet between the proline-rich sequence,  $\beta x$ , and residues 458–461 in the carboxy-terminal half of RPTP $\alpha$ D1,  $\beta y$ , has no structural counterpart in PTP1B. PTP1B lacks the N-terminal extension encompassing the prolines and contains a helical three-residue insertion in the second segment, located towards the C terminus.

Using the consensus sequence for the N-terminal structural wedge (residues 208–242) generated from a small subset of D1 sequences illustrated in Fig. 3*b*, we retrieved all available D1 primary sequences from a database search. In contrast, no D2 catalytic domains of RPTPs or catalytic domains of non-receptor PTPs were retrieved. Consequently, we propose that this structurally constrained N-terminal wedge is a functionally conserved motif present in the large family of RPTPs. The sites for protein kinase C (PKC)-mediated phosphorylation of RPTP $\alpha$ , Ser 180 and Ser 204, reside near the dimer interface, suggesting a possible role for PKC-mediated phosphorylation in the regulation of RPTP $\alpha$  oligomerization<sup>13</sup>.

A question that remains is whether other RPTPs dimerize like D1. The existence of the conserved N-terminal extension is consistent with the possibility that all RPTPs can dimerize and the variable residues in this region could provide the necessary dimerization specificity. Experimentally, there is evidence that CD45, a PTP required for antigen-dependent activation of T cells through the T-cell receptor, is negatively regulated by dimerization. The existence of CD45 homodimers has been detected using a dithio-bis-succinimidyl propionate crosslinking agent in YAC-1 cell lysates<sup>14</sup>. Moreover, epidermal growth factor (EGF)-induced dimerization of an artificial EGF receptor (extracellular and transmembrane portion)/CD45 (intracellular portion) chimera expressed in a CD45-deficient T-cell line causes loss of antigen-dependent activation, whereas co-expression of the EGF receptor/CD45 chimera gene with the gene encoding only the extracellular and transmembrane portions of the EGF receptor restored T-cell signalling even in the presence of an EGF receptor ligand<sup>3</sup>. Given that phosphatase activity of the D1 domain of CD45 is required for antigen-dependent activation, EGF-induced dimerization of the intracellular portion of CD45 is likely to occur in a manner analogous to that of the RPTP $\alpha$ D1 dimer described herein, thus explaining the loss of catalytic activity.



FIG. 3 Comparisons of RPTP $\alpha$ D1 with other tyrosine phosphatases. *a*, Main-chain representation of the N-terminal wedge and residues 456 to 463 (238 to 248 in PTP1B) of RPTP $\alpha$ D1 and PTP1B. D1 is grey. PTP1B (Brookhaven Protein Data Bank code 2HNP) is orange. Side chains of D1 residues comprising the consensus RPTP N-terminal wedge are included. Many of the conserved residues in D1 define the conformation of the wedge. In particular, a cluster of hydrophobic contacts provided by the side chains of Leu 217, Ile 221, and Phe 232, and the methylenes of Arg 224 and Glu 235 structurally define the interhelical angle. A salt bridge connecting Arg 224 to Glu 235 acts as a tether across the interhelical wedge. The Pro-Pro-Leu-Pro sequence (residues 210–213) forms a two-stranded  $\beta$ -sheet with residues 457 to 462 of RPTP $\alpha$ D1. This rigid structural element closes off the amino-terminal wedge and anchors it to the rest of the molecule. Produced with the conic option<sup>24</sup> of MIDAS<sup>25</sup>. *b*, Sequence alignment of the N-terminal wedge of RPTPs' membrane-proximal catalytic domains<sup>27</sup>. Line one schematically illustrates the secondary structural elements observed in RPTP $\alpha$ D1. Line two gives the consensus sequence conserved across the large family of D1 domains. The numbering scheme refers to the short variant of murine RPTP $\alpha$ <sup>28</sup> with the first residue of the signal peptide numbered as 1. The stars indicate residues involved in active-site-directed dimeric interactions. Residues boxed in black are common to at least five aligned D1 sequences. (*m* is mouse, *h* is human, *r* is rat, and *d* is *Drosophila*). The first 14 homologous sequences contrast sharply with the lack of sequence conservation in the final segments. The most distinguishing feature of the RPTP N-terminal wedge involves a two-amino-acid insertion (Asp 227 and Asp 228) into the tip of the non-receptor-like



PTP N-terminal segment. The second to the last line corresponds to the numbering scheme of human PTP1B. The final line emphasizes the structural elements observed in the PTP1B crystal structure<sup>8</sup>.

TABLE 1 Refinement statistics for crystal form P2<sub>1</sub>

| Resolution shell (Å)          | 4.29                 | 3.50  | 3.08  | 2.81  | 2.62             | 2.47  | 2.35  | 2.25  | Total  |
|-------------------------------|----------------------|-------|-------|-------|------------------|-------|-------|-------|--------|
| Number of reflections         | 3,331                | 3,312 | 3,268 | 3,256 | 3,233            | 2,931 | 1,799 | 564   | 21,694 |
| Per cent complete             | 99.9                 | 99.6  | 99.0  | 99.1  | 97.5             | 89.8  | 56.6  | 17.4  | 82.4   |
| $\langle I/\sigma(I) \rangle$ | 34                   | 28    | 23    | 17    | 13               | 10    | 8     | 7     | 22     |
| $R_{\text{sym}}^*$            | 0.042                | 0.051 | 0.038 | 0.040 | 0.056            | 0.067 | 0.091 | 0.104 | 0.046  |
| $R$ -factor $\pm$             | 0.244                | 0.159 | 0.180 | 0.191 | 0.213            | 0.230 | 0.240 | 0.266 | 0.202  |
| Free $R$ -factor $\pm$        | 0.277                | 0.206 | 0.264 | 0.269 | 0.292            | 0.296 | 0.301 | 0.300 | 0.262  |
| R.m.s. deviations             | Bond lengths 0.009 Å |       |       |       | Bond angles 1.4° |       |       |       |        |

Truncated D1 (residues 202–503) was obtained by digestion of immobilized GST–RPTP $\alpha$ D1, residues 167 to 503 (ref. 5) with 1 mg of trypsin per 50 mg of GST–RPTP $\alpha$ D1 in 50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM dithiothreitol, for 1 h at 4 °C. D1 was further purified with a Pharmacia MonoQ column (pH 8.0, NaCl gradient). D1 crystals were grown by vapour diffusion in hanging drops at a protein concentration of 15 to 20 mg ml<sup>-1</sup> in 50 mM sodium acetate, pH 5.0, 0.3 M ammonium acetate, 12% (w/v) PEG 8000. Crystals were flash-frozen with 20% (v/v) PEG 400. Diffraction data were collected on a DIP2000 imaging plate system equipped with double focusing Pt/Ni coated mirrors (Mac-Science Corporation, Japan). Data were processed with DENZO<sup>15</sup>, scaled with SCALEPACK<sup>15</sup> and further processed with CCP4 programs<sup>16</sup>. Molecular replacement was performed in the space group C222<sub>1</sub> ( $a = 42.3$  Å,  $b = 115.4$  Å,  $c = 119.9$  Å, one molecule per asymmetrical unit) using a trimmed model of PTP1B (PDB entry code 2HNP; 34% sequence identity) with AMoRe (ref. 17) integrated in an AVS environment<sup>18,19</sup>. After rigid-body refinement (one body) the  $R$ -factor dropped to 0.46 (data 20.0 to 4.5 Å). The model was adjusted using O (ref. 20) and refined with X-PLOR (ref. 21). Higher quality crystals were then obtained (same conditions) in the related space group P2<sub>1</sub> ( $a = 42.2$  Å,  $b = 119.8$  Å,  $c = 61.2$  Å,  $\beta = 110.1^\circ$ , two molecules per asymmetrical unit). In this crystal form, the second molecule of the dimer is generated by a non-crystallographic two-fold which corresponds to the crystallographic two-fold parallel to  $a$  of C222<sub>1</sub>. X-PLOR refinement of the dimer and subsequent introduction of water molecules using the ARP software<sup>22</sup> allowed the unambiguous modelling of residues 209 to 217. The current model comprises 2  $\times$  280 residues (Lys 208–Tyr 495) and 395 ordered water molecules. Residues 379 to 386 are disordered in the model; secondary tryptic cleavage sites exist at Arg 385 and Lys 386. The Ramachandran plot<sup>23</sup> of the final model shows that 89% of the residues are in most favoured regions. The final root-mean-square-deviation between the search model and the refined structure is 1.0 Å for 246 C $\alpha$  atoms deviating by less than 3.5 Å. A second crystal form of D1 (P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>,  $a = 80.0$  Å,  $b = 116.5$  Å,  $c = 42.2$  Å, one molecule per asymmetrical unit) was grown using the same crystallization conditions as described above in the presence of an excess of the non-hydrolysable phosphonate ester analogue of the peptide used in the PTP1B–peptide complex<sup>11</sup>. Diffraction data to 1.7 Å were collected at the Stanford Synchrotron Radiation Laboratory, beamline 7–1 ( $\lambda = 1.08$  Å) on an MAR imaging plate system. Data processing was performed as described previously. The second crystal form was solved using the D1 model with AMoRe. After rigid-body refinement, the  $R$ -factor was 36% (data 20.0 to 3.2 Å). Further refinement is in progress. So far, no electron density for the peptide has been observed.

\*  $R_{\text{sym}} = \sum_h |I_h - \langle I_h \rangle| / \sum_h I_h$ , where  $\langle I_h \rangle$  is the average intensity of reflection  $h$  for its symmetry and Friedel equivalents.  $\pm R$ -factor =  $\sum_h (|F_o(h) - F_c(h)|) / \sum_h F_o(h)$ . Free  $R$ -factor is calculated using 10% of the data that has never been used for refinement. Unless specified, the  $R$ -factor is calculated using the remaining 90% of the data with no  $\sigma$  cutoff.

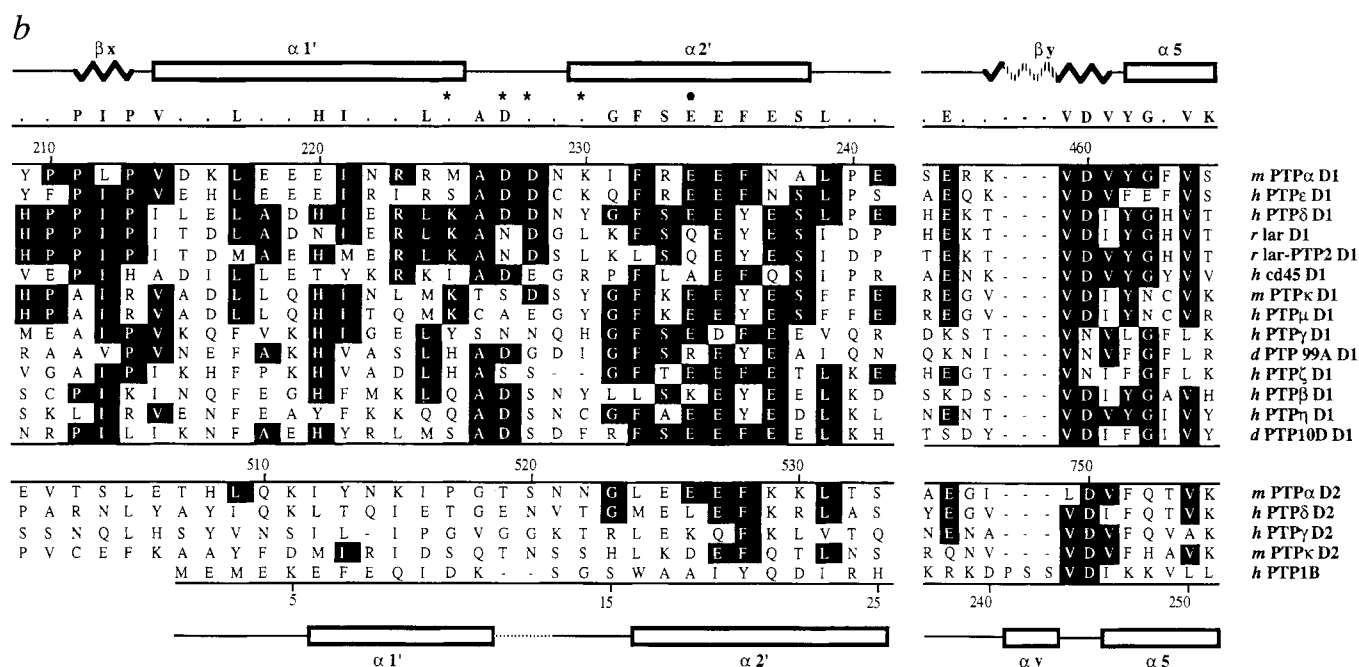
The two independent RPTP $\alpha$ D1 crystal structures, the primary sequence conservation of the dimer interface, and the CD45/EGF chimera study<sup>3</sup> link the oligomeric structure of RPTP $\alpha$ D1 with a simple regulatory scheme in which dimerization inhibits RPTP $\alpha$  activity. As such, it should serve as a model for the functional and structural features governing the negative regulation of RPTP activity. □

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**CORRESPONDENCE** and requests for materials should be addressed to J.P.N. (e-mail: noel@sbl.salk.edu). Coordinates for the RTPTαD1 dimer will be submitted to the Brookhaven Protein Data Bank.

## Recognition of DNA by designed ligands at subnanomolar concentrations

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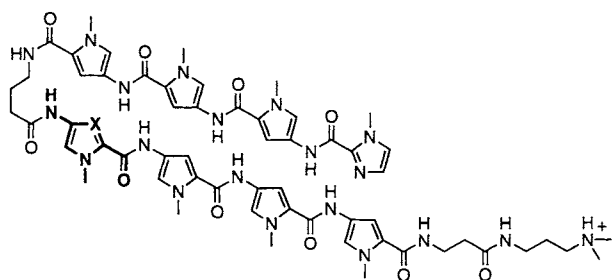
**SMALL molecules that specifically bind with high affinity to any predetermined DNA sequence in the human genome would be useful tools in molecular biology and potentially in human medicine. Simple rules have been developed to control rationally the sequence specificity of minor-groove-binding polyamides containing *N*-methylimidazole and *N*-methylpyrrole amino acids. Two eight-ring pyrrole–imidazole polyamides differing in sequence by a single amino acid bind specifically to respective six-base-pair target sites which differ in sequence by a single base pair. Binding is observed at subnanomolar concentrations of ligand. The replacement of a single nitrogen atom with a C-H regulates affinity and specificity by two orders of magnitude. The broad range of sequences that can be specifically targeted with pyrrole–imidazole polyamides, coupled with an efficient solid-phase synthesis methodology, identify a powerful class of small molecules for sequence-specific recognition of double-helical DNA.**

For side-by-side complexes of pyrrole–imidazole polyamides in the minor groove of DNA, the DNA-binding sequence specificity depends on the sequence of side-by-side amino-acid pairings<sup>1–3</sup>. A

pairing of imidazole (Im) opposite pyrrole (Py) targets a G·C base pair, whereas pyrrole opposite imidazole targets a C·G base-pair<sup>1–3</sup>. A pyrrole/pyrrole combination is degenerate and targets both T·A and A·T base pairs<sup>1–5</sup>. Specificity for G·C base pairs results from the formation of a hydrogen bond between the imidazole N3 and the exocyclic amino group of guanine<sup>1–3</sup>. The generality of these pairing rules has been demonstrated by targeting a wide variety of sequences and is supported directly by several NMR structure studies<sup>1–10</sup>.

In parallel with the elucidation of the scope and limitations of the pairing rules already described, efforts have been made to increase the DNA-binding affinity and specificity of pyrrole–imidazole polyamides by covalently linking polyamide subunits<sup>11–16</sup>. The polyamide ImPyPy-γ-PyPyPy-dimethylaminopropylamide (Dp), which contains a 'turn' amino acid γ-aminobutyric acid (γ) was shown to bind the five-base-pair target site 5'-TGTTA-3' in a 'hairpin' conformation with an equilibrium association constant,  $K_a = 8 \times 10^7 \text{ M}^{-1}$ , an increase of 300-fold relative to unlinked three-ring polyamide dimers. A key issue was to determine whether low-molecular-weight ( $M_r$  1,200) pyrrole–imidazole polyamides could be constructed that would bind DNA at subnanomolar concentrations without compromising sequence selectivity.

We report the DNA-binding affinities of two eight-ring hairpin polyamides, ImPyPyPy-γ-ImPyPyPy-β-Dp (1) and ImPyPyPy-γ-PyPyPyPy-β-Dp (2), which differ by a single amino acid, for two 6-base-pair (bp) target sites, 5'-AGTACT-3' and 5'-AGTATT-3', which differ by a single base pair. Based on the pairing rules for polyamide–DNA complexes, the sites 5'-AGTACA-3' and 5'-AGTATT-3' are for polyamide 1 'match' and 'single-base-pair mismatch' sites, respectively, and for polyamide 2 'single-base-pair mismatch' and 'match' sites, respectively (Figs 1 and 2).



- 1** X=N; ImPyPyPy-γ-ImPyPyPy-β-Dp  
**2** X=CH; ImPyPyPy-γ-PyPyPyPy-β-Dp

FIG. 1 Structures of polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp (**1**) and ImPyPyPy-γ-PyPyPyPy-β-Dp (**2**). (Im = imidazole, Py = pyrrole, β = β-alanine, Dp = dimethylaminopropylamide). The identity and purity of the polyamides was verified by  $^1\text{H}$  NMR, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and analytical HPLC. MALDI-TOF MS: **1**, 1223.4 (1223.3 calculated); **2**, 1222.3 (1222.3 calculated).

Polyamides **1** and **2** were synthesized by solid-phase methods and purified by reversed-phase high-performance liquid chromatography (HPLC)<sup>17</sup>. Equilibrium association constants for complexes of **1** and **2** with match and mismatch six-base-pair binding sites on a 3'  $^{32}\text{P}$ -labelled 229-bp restriction fragment were determined by quantitative DNase I footprint titration experiments<sup>18-20</sup> (Fig. 3 and Table 1). Polyamide **1** binds its match site 5'-AGTACT-3' at 0.03 nM concentration and its single-base-pair mismatch site 5'-AGTATT-3' with nearly 100-fold lower affinity. Polyamide **2** binds its designated match site 5'-AGTATT-3' at 0.3 nM concentration and its single-base-pair mismatch site 5'-AGTACT-3' with nearly 10-fold lower affinity. The specificity of **1** and **2** for their respective match sites results from very small structural changes (Fig. 1). Replacing a single nitrogen atom in **1** with C-H (as in **2**) reduces the affinity of the polyamide-5'-AGTACT-3' complex by ~75-fold, representing a free energy difference of ~2.5 kcal mol<sup>-1</sup>. Similarly, replacing a C-H in **2** with N (as in **1**) reduces the affinity of the polyamide-5'-AGTATT-3' complex ~10-fold, a loss in binding energy of ~1.3 kcal mol<sup>-1</sup>.

Crystal structures of protein-DNA complexes reveal that nature chose a combinatorial approach for specific DNA recognition. Although there are several highly conserved structural

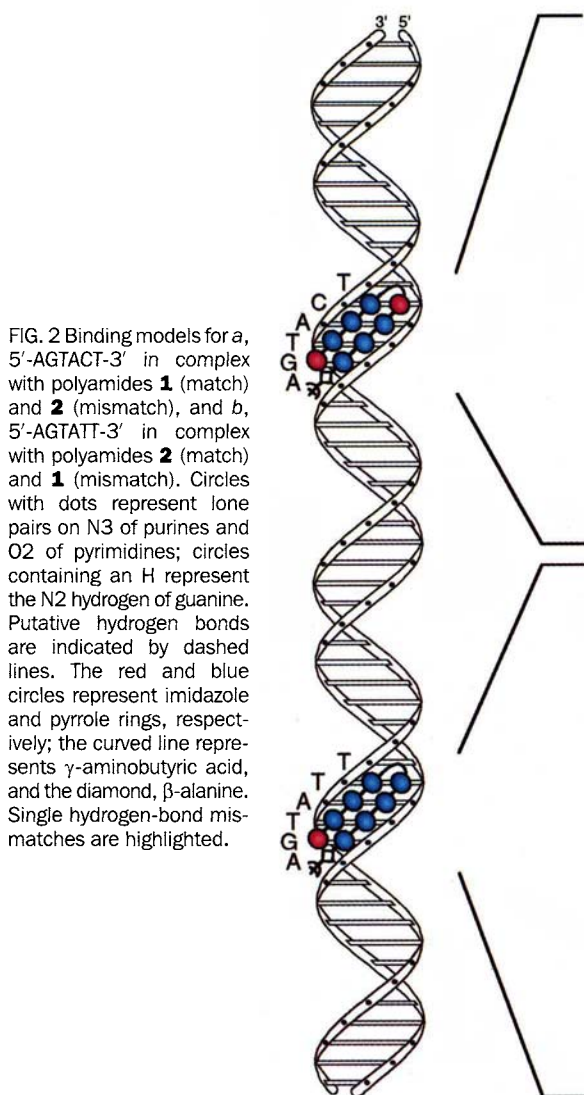
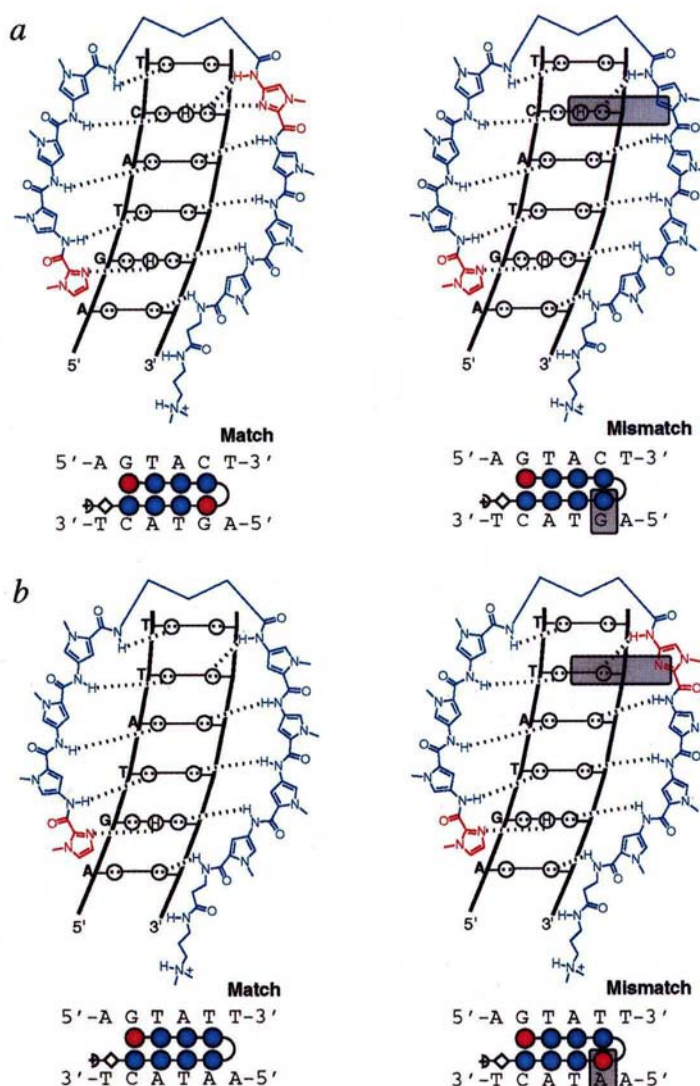


FIG. 2 Binding models for a, 5'-AGTACT-3' in complex with polyamides **1** (match) and **2** (mismatch), and b, 5'-AGTATT-3' in complex with polyamides **2** (match) and **1** (mismatch). Circles with dots represent lone pairs on N3 of purines and O2 of pyrimidines; circles containing an H represent the N2 hydrogen of guanine. Putative hydrogen bonds are indicated by dashed lines. The red and blue circles represent imidazole and pyrrole rings, respectively; the curved line represents γ-aminobutyric acid, and the diamond, β-alanine. Single hydrogen-bond mismatches are highlighted.



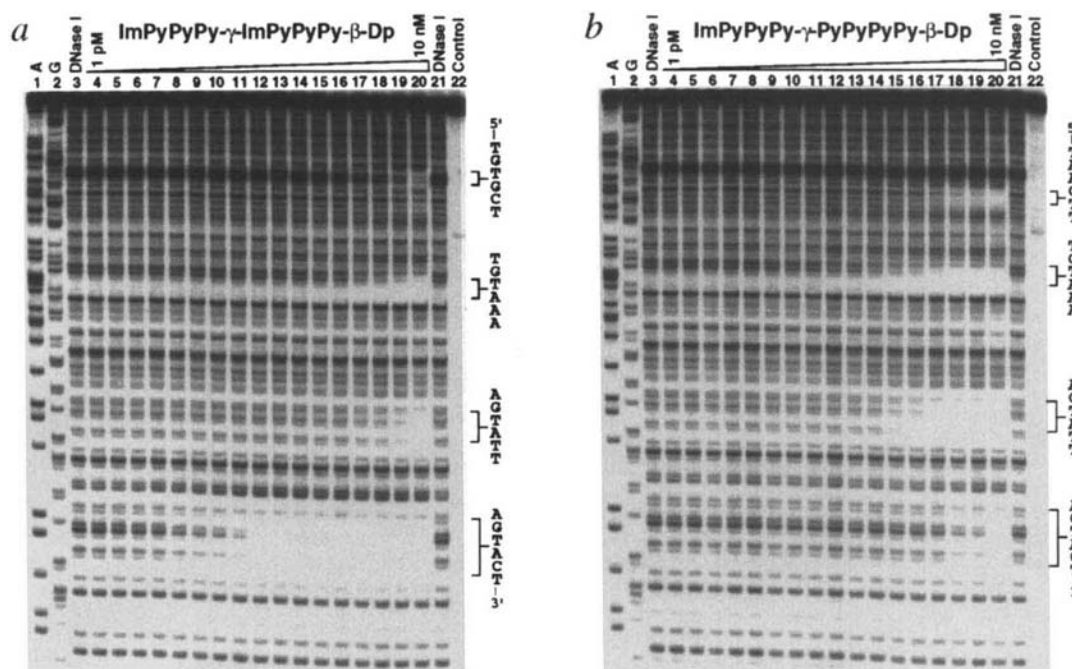


FIG. 3 Quantitative DNase I footprint titration experiments with polyamides **1** and **2** on the 3' <sup>32</sup>P-labelled 229-bp pJT8 *Afl*/I/*Fsp*I restriction fragment. Lanes 1 and 2: A and G sequencing lanes<sup>29,30</sup>; lanes 3 and 21: DNase I digestion products in the absence of polyamide; lanes 4–20: DNase I digestion products in the presence of 1, 2, 5, 10, 15, 25, 40 and 65 pM, and 0.1, 0.15, 0.25, 0.4, 0.65, 1, 2, 5 and 10 nM polyamide, respectively; lane 22: intact DNA. Polyamide binding sites for which association constants were determined are 5'-AGTACT-3' and 5'-AGTATT-3'. Additional sites not analysed are 5'-TGTAAG-3', 5'-TGTGCT-3' and 5'-TAAGTT-3'. All reactions were done in a total volume of 400  $\mu$ l. A polyamide stock solution or H<sub>2</sub>O was added to an assay buffer containing radiolabelled restriction

fragment, with final solution conditions of 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, pH 7.0. Solutions were allowed to equilibrate for 12–15 h at 22 °C before initiation of footprinting reactions. Footprinting reactions, separation of cleavage products, and data analysis were carried out as described<sup>24</sup>. Plasmid pJT8 was prepared by hybridizing two 5'-phosphorylated complementary oligonucleotides, 5'-CCGGTTAGTATTGG-ATGGGCTCGGTAGTACTTGGATGGAGACCGCCTGGGAATACCAGGTGTCGATC-TTAAGAG-3' and 5'-TCGACTCTTAAGATACGACACCTGGTATTCAGGCGGTC-TCCCATCCAAGTAAACACGAGGCCATCCAAATCTAA-3', and ligating the resulting duplex to the large pUC19 *Ava*/I/*Sa*I restriction fragment.

TABLE 1 Equilibrium association constants (M<sup>-1</sup>)

| Binding site     | <b>1</b>                   | <b>2</b>                |
|------------------|----------------------------|-------------------------|
| 5'-ttAGTACTtg-3' | $3.7 \times 10^{10}$ (0.8) | $5.0 \times 10^8$ (0.5) |
| 5'-ttAGTATTtg-3' | $4.1 \times 10^8$ (0.5)    | $3.5 \times 10^8$ (0.8) |

The association constants are the average values obtained from three DNase I footprint titration experiments. The standard deviation for each data set is indicated in parentheses. Assays were carried out in the presence of 10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl<sub>2</sub> and 5 mM CaCl<sub>2</sub> at pH 7.0 and 22 °C. The six-base-pair binding sites are in capital letters, with flanking sequences in lower-case.

modules that bind DNA, no single motif exists which generates an amino acid–base pair code for all DNA sequences<sup>21</sup>. One encouraging success is the development of zinc-finger libraries with different DNA-binding specificities based on a one-finger–three-nucleotide code<sup>22–27</sup>. The three-zinc-finger DNA-binding domain of the transcription factor Zif268 has a dissociation constant  $K_d = 0.5$ – $3.0$  nM for its 9-bp binding site and is 2–12-fold specific over single-base-pair mismatches<sup>24–27</sup>. Similarly, the three-zinc-finger transcription factor Sp1 has a  $K_d = 0.5$  nM for its 9-bp recognition sequence and is 3–30-fold specific over single-base-pair mismatches<sup>28</sup>. Using a simple molecular shape and a two-letter aromatic amino-acid code, pyrrole–imidazole polyamides achieve affinities and specificities comparable to these DNA-binding proteins and, in addition, have the potential to be general for any desired DNA sequence. This non-biological approach to DNA recognition could provide an underpinning for the design of cell-permeable molecules for the control of gene-specific regulation *in vivo*. □

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